ABSTRACT

A neoplastic disease that affects a common species of marine fish, the bicolor damselfish (Poracentrus partitus), on Florida reefs consists of multiple, disseminated neurofibromas (including plexiform lesions), malignant schwannomas, and hyperpigmented epidermal lesions. Based on similarities to von Recklinghausen neurofibromatosis, we have termed this disease damselfish neurofibromatosis. Previous surveys of the prevalence of fish with damselfish neurofibromatosis on Florida reefs demonstrated a distribution pattern of cases consistent with what would be expected for an infectious disease. The transmissibility of damselfish neurofibromatosis was assayed by inoculations of homogenized tumor tissue s.c. and i.p. into healthy bicolor damselfish. This protocol resulted in the development of Schwann cell tumors, identical to the naturally occurring lesions, at the injection sites in approximately 84% of inoculated fish. These tumors appeared within an average of 5.5 mo of inoculation for juvenile fish and 14 mo for adults. Experimentally produced tumors appeared to arise in host fish by the neoplastic transformation of host nerves rather than by transplantation and proliferation of tumor cells from the donor fish. This finding suggests that an infectious, transmissible agent such as a virus may be the etiological agent responsible for production of neurofibromas and other Schwann cell tumors in this species of fish.

INTRODUCTION

We have previously described a neoplastic disease that affects a common species of marine fish, the bicolor damselfish (Poracentrus partitus), on Florida reefs (1, 2). The histopathological features of this disease are similar to many of the major stigmata of von Recklinghausen NF3 including: multiple, disseminated neurofibromas (including plexiform lesions); malignant schwannomas; and hyperpigmented epidermal lesions. Based on these similarities, we have termed this disease DNF. The human and fish diseases differ chiefly in that the fish tumors exhibit a higher degree of malignancy, and the hyperpigmented lesions are composed primarily of neoplastic, rather than benign, cells.

Neurofibromatosis in humans has been established to be an autosomal dominant disorder with essentially complete penetrance. However, approximately one-half of the known cases is attributed to new mutations (3). Considering the high incidence of this disease (approximately 1 in 3000 births), this represents one of the highest mutation rates reported for a genetic disease (3, 4). Surveys for oncogene expression have not yielded positive results (5), and the gene or genes responsible for this disorder have not been identified. However, recent DNA restriction fragment length polymorphism studies of affected families have suggested that at least one gene involved in von Recklinghausen NF is located on chromosome 17, and the gene responsible for the central form of NF is located on chromosome 22 (6, 7). The lack of information on the actual gene(s) or gene products responsible for this disorder as well as a lack of understanding of the extreme variability in phenotypic expression seen in various forms of NF may be largely attributable to the absence of suitable animal models of NF. Although Schwann cell tumors have been observed sporadically in various mammalian species, none appears predictably or at high incidence. Malignant schwannomas have been produced in neonatal rats by transcriptional administration of nitrosamines. However, these animals do not develop neurofibromas or other signs of NF. Recently, neurofibromas have been observed in transgenic mice produced by the insertion of the tat gene and associated long terminal repeat of HTLV-1 into fertilized eggs (8). Numerous cases of peripheral nerve sheath tumors have been reported in fishes (9–11). However, most of these cases represent isolated occurrences, and attempts to transfer such tumors have been unsuccessful (12). At present, there are apparently no documented cases of successful transmission of a Schwann cell tumor to a noninbred or nonimmunosuppressed host.

Surveys of the prevalence of fish with DNF conducted previously on reefs in the Florida Keys demonstrated that diseased fish were significantly more abundant on reef areas with higher population densities of P. partitus and that affected fish were spatially clustered (13). These results are consistent with distribution patterns produced by infectious diseases. Bicolor damselfish are aggressive, territorial fish that often inflict injuries on each other in the course of territorial conflicts. Thus, the social behavior of these fish would provide ample opportunity for the contact transmission of an infectious agent. An experimental protocol was designed to assess the potential for transmission of DNF to healthy bicolor damselfish. This protocol was designed to maximize the likelihood of successfully transferring the tumors.

MATERIALS AND METHODS

Bicolor damselfish used in these studies were collected from reefs offshore of southern Florida. Fish were maintained in the laboratory in 110-liter aquaria, subdivided into 37-liter compartments holding one fish each. Experimental and control fish were kept in different aquaria. Aquaria were maintained as closed systems at 21–27°C at a salinity of 30–38 ppt. All fish were observed in the laboratory for at least 2 wk prior to experiments to ascertain that they were asymptomatic at the time an experiment began.

Five transmission experiments were conducted utilizing a total of 43 fish. Two control studies involved 11 fish. Adult fish of an approximately equal sex ratio as well as juveniles were utilized in these studies. Gonadal maturation occurs in P. partitus when fish are 55 to 65 mm in total length (14). In the present study, fish 60 mm and larger were considered to be adults. These fish reach a maximum length of about 85 mm.

Transmission experiments were conducted by injecting a homogenate of tumor tissue into healthy fish. In each experiment, except Experiment 2, both pigmented and nonpigmented tumor tissues were obtained from one or two fish with spontaneous DNF. A serial passage of the tumor was attempted in Experiment 2, with donor tissue being obtained from a nonpigmented tumor that developed at an injection site of a
fish from Experiment 1. Control fish were given injections of an equal quantity of a homogenate produced from lateral body musculature of healthy fish. This choice of control tissue represented a compromise, as no direct, normal analogue to the Schwann cells of the tumors was available. Tissue from the central nervous system was not used as a control in order to avoid the possibility of producing an autoimmune encephalitis.

Appropriate tissues were removed under sterile conditions from donor fish euthanized with MS-222 (Tricain methane sulfonate). Cell suspensions were made by grinding tissue mixed with sterile Eagle’s MEM (Flow Laboratories) with Hanks’ salts in a Ten Broek tissue homogenizer over ice. Tissue was mixed with MEM prior to homogenization at a ratio of 40 to 150 mg of tissue per ml of MEM. These tumor tissues contained variable amounts of live tumor cells, necrotic tumor tissue, and extracellular collagen. This variability prevented the calculation or close control of the number of tumor cells or the weight of viable tumor cells used in a preparation. Samples of homogenates from both tumor and normal muscle tissue were found to contain less than 20% intact cells using a trypan blue exclusion technique.

Experimental and control fish were given injections at three sites, two s.c. and one i.p. Injection volumes were adjusted to the size of each subject, such that the amount of homogenate retained was maximized while minimizing runoff from the s.c. sites. Adult fish (60 to 85 mm in total length) received approximately 50 μl of homogenate per site, and juvenile fish (less than 60 mm) received 15 to 30 μl per site. Injection volumes (i.p.) were matched to those used for s.c. sites in each fish.

Experimental fish were observed for periods of 12 to 24 mo following inoculation. Fish in control Studies 1 and 2 were observed for 29 and 14 mo, respectively. Observations on surviving fish in Experiments 3 to 5 and control Study 2 were continuing as of the time that this report was submitted. All experimental and control fish were preserved in 10% buffered formalin at the termination of each experiment or as close as possible to the time of death of fish that died during the observation period. Selected fish that developed externally visible lesions (nodules or hyperpigmented spots) were embedded in paraplast, sectioned at 7 μm, stained with hematoxylin-eosin or Luxol Fast Blue, and examined to compare histological features with those observed in naturally occurring DNF. All fish in Experiments 1 and 2 that failed to exhibit development of externally visible lesions or showed questionable development and all fish from the first control study were sectioned and examined to determine if any type of neoplastic tissue was present.

RESULTS

Indications of tumor development were first observed as hyperpigmented spots approximately 1 mm in diameter near the s.c. injection sites. These spots increased in area and relief-hyperpigmented regions 5 to 10 mm in diameter (Fig. 1a). Often, nonpigmented nodules of 1 to 3 mm in relief were also present at these sites (Fig. 1b). Lesions followed similar initial patterns of development in all experimental fish that developed tumors.

These lesions were similar in gross appearance to those observed in fish naturally affected with DNF (see Refs. 1 and 2 for comparison). Those involving the dermis and epidermis were typically hyperpigmented and were accompanied by erosion and distortion (bony hyperplasia) of adjacent scales. Lesions occurring in s.c. areas were visible as nonpigmented nodules, often erupting through the skin. Initial development was always restricted to the immediate areas (within 5 to 10 mm) of the inoculation sites, although not all sites exhibited changes in all fish. Several fish also exhibited subsequent discontinuous spread to other areas of the body and fins.

In the second experiment tumors were serially passaged by using a tumor which developed in the first experiment as donor tissue. The tumors which arose in these fish were grossly and microscopically identical to those described above. In addition, although no pigmented tumor material was injected in the second experiments, several fish developed pigmented tumors at the injection sites. This indicates that both pigmented and nonpigmented tumors may arise following injection of a homogenate of nonpigmented tumor cells.

Histological examination of experimental fish demonstrated that these lesions were identical in cell morphology and patterns of organization to the tumors observed in naturally occurring DNF. The experimentally produced tumors were composed of varying proportions of elongate, spindle-shaped cells arranged in parallel, whorling bundles with interlacing fascicles and areas of plump, pleomorphic cells (Fig. 2). Tumors that developed at the sites of i.p. injections appeared to originate in the body wall rather than in the peritoneal cavity or the viscera. There was no evidence of any ascites tumor formation. Tumors were never observed to be encapsulated. These patterns are typical of those observed in the naturally occurring tumors (1, 2). These findings demonstrate that the Schwann cell tumors involved in DNF are transmissible to healthy, noninbred fish.

Several experimentally produced tumors were examined in the early stages of development to document the histology of these early masses of neoplastic cells and their relationship to adjacent tissues. These tumors were observed to be in close contact with multiple branches of peripheral nerves adjacent to the injection sites (Fig. 3a). Such nerves were abnormal in appearance and were characterized by an increase in the diameter of individual myelin sheaths throughout the nerve leading to an increase in the overall diameter of the nerve accompanied by a disorientation of the normal, parallel arrangement of axons (Fig. 3, b and c). This indicated that these nerves were also abnormally elongated, producing a partial folding of regions of the nerve bundle. An increase in collagen content was also observed in these nerves. Staining of these regions with Luxol Fast Blue confirmed that the abnormally enlarged nerves were...
still well myelinated and that tumors immediately adjacent to these nerves contained sparsely myelinated cells. This histological pattern is typical of that observed in the early stages of formation of plexiform neurofibromas in humans (15) and in DNF in affected nerves in the initial stages of neoplastic transformation. These observations indicated that some nerves near the inoculation sites in experimental fish were undergoing transformation.

Tumors exhibited invasive, malignant growth patterns, characterized by the infiltration of tendrils of tumor cells into the surrounding tissues, even in the earliest stages that lesions could be detected microscopically. Histological features in some regions were similar to those encountered in malignant schwannomas in humans and other animals. Tumor cells and normal tissue were visible as distinct regions at interface areas where destruction of normal tissues, including skeletal muscle, connective tissue, and bone, could be observed. In contrast, zones of interface between tumors and adjacent abnormal nerves exhibited multiple areas of gradual transition between identifiable nerve fibers and neoplastic cells. These histological patterns suggest that tumors were arising from these peripheral nerves rather than infiltrating them.

Following initial appearance at injection sites, tumors gradually spread to cover larger areas of the body contiguous with the original tumor. Disseminated lesions, distant from the initial tumors, were observed in 39% of the fish that developed tumors. Disseminated tumors were histologically identical to the primary tumors and appeared to originate in local nerves. A case was observed of such a tumor arising in a branch of the trigeminal nerve adjacent to the mandible. This was one of the...
the latency of tumor development, or the elapsed time between inoculation and the appearance of a tumor. Observations on the size, external appearance, and rate of growth of tumors were not quantified in this study.

None of the fish in the control studies showed evidence of tumor development. Tumors were observed in 84% (16 of 19) of the adult and 83% (20 of 24) of the juvenile experimental fish. The 3 adult fish counted as nontumored might have developed tumors if allowed a longer incubation period. However, these animals were sacrificed 10 mo following injection to screen them for microscopic tumor masses. The 4 juvenile fish without visible lesions were monitored for a minimum of 20 mo. Based on tumor latency times observed in juvenile fish (see below), tumor development was not likely to have occurred in these fish at a later time. The frequency of positive takes did not vary significantly between experiments or between adult and juvenile fish.

Times for latency of tumor development ranged from 6 wk to 27 mo (Fig. 4). All but 3 of the 36 fish exhibiting tumors had latency periods of less than 15 mo. A highly significant difference (T test statistic, $P < 0.01$) was observed in mean latency times between adult (mean, 14.0 mo; 1 SD = 6.0) and juvenile fish (mean, 5.3 mo; 1 SD = 2.9). There was little overlap in the latency periods with all juvenile fish developing tumors within 10 mo of injection, while all but 3 of 16 adult fish showed latency periods greater than 10 mo (Fig. 4). Thus, the relationship between size and tumor latency was a discontinuous function, reflecting an abrupt change in latency observed in fish that were sexually mature (that is, greater than 60 mm in total length) at the time of injection. Within the juvenile and adult size categories, no correlations between host size and latency were observed. No differences were observed in frequency or latency between males and females.

Most fish developed tumors at several of the injection sites, with 61% showing involvement of at least two sites and 31% exhibiting lesions at all three sites. There was no difference in the average number of sites involved in adult versus juvenile fish. The two s.c. injection sites were more frequently involved and also exhibited a somewhat shorter average latency period than the i.p. sites.

**DISCUSSION**

The tumors that developed in the experimental fish were identical histologically to those seen in fish naturally affected

![Fig. 3. Relationship of experimentally produced tumors near a s.c. injection site to local nerves. In a, an abnormal, enlarged nerve is visible (arrow) surrounded by neoplastic cells. Tumor cells can also be seen invading adjacent musculature (arrowhead). Areas of hyperpigmented tumor cells (b) are also present immediately below several scales (s). H & E; bar, 20 μm. b, higher magnification of abnormal nerve in Fig. 3a. Note numerous enlarged myelinated axons (examples at arrow), loss of parallel arrangement of individual fibers, and an overall enlargement of the nerve bundle when compared to a normal nerve (Fig. 3c). H & E; bar, 5 μm. c, normal counterpart of nerve shown in Fig. 3, a and b, located on the opposite side of body in an identical position to this nerve, identical magnification to that used in b. Note small, myelinated axon (arrow). H & E; bar, 5 μm.](https://cancerres.aacrjournals.org/fig3)

![Fig. 4. Relationship of tumor latency to size of fish receiving injection. Only fish developing tumors following inoculation are shown (n = 36). A highly significant difference (t test; $P < 0.001$) in mean latency of tumor development was observed between juvenile (x) and adult (c) fish.](https://cancerres.aacrjournals.org/fig4)
with DNF, demonstrating that these Schwann cell tumors are transmissible to noninbred, nonimmunocompromised hosts. We believe that this is the first reported case of a transmissible Schwann cell tumor. Schlumberger (12) reported similar tumors in goldfish which, except for a single autograft, were not transplantable. These results also demonstrate the potential for fish-to-fish transmission of these tumors in nature.

The tumor development observed in the experimental fish could have been produced by at least three mechanisms. Trauma to local nerves as a result of the injections could have produced neoplastic masses or traumatic neuromas (15). However, the absence of tumor development in any of the control fish demonstrated that the trauma of injecting the cell homogenates s.c. or i.p. was insufficient to produce tumors of any type in this species. Alternatively, neoplastic cells that survived the homogenization process and escaped immune surveillance in the new host could have established colonies of proliferating cells yielding tumors. Finally, some agent in the tumor homogenate, such as a chemical carcinogen, an oncogenic virus, or transfected DNA from donor cells, could have induced neoplastic transformation of Schwann cells in the nerves of the host fish, producing tumors. Cell transformation via transfection of DNA from neoplastic cells has been reported in vitro but has not been described from any tumor systems in vivo (16).

The possibility of tumor production via transplantation of viable cells is a function of the ability of such cells to escape immune surveillance in the new host. Such escape would result primarily from the absence of recognizable foreign antigens on transplanted cells and/or the inability of the fish's immune system to destroy such cells.

The presence of polymorphic histocompatibility antigens is generally assumed in samples of most vertebrate animals from noninbred populations. The life cycle of most marine fishes, including P. partitus, involves a pelagic larval stage. A pelagic stage results in the mixing and transport of larvae by ocean currents over large areas, such that little or no genetic similarity exists between neighboring fish on a given reef (17). Thus, an essentially panmictic distribution of genotypes can be assumed for P. partitus, with no possibilities for inbreeding to occur in natural populations.

The ability of P. partitus to recognize and destroy cells with foreign antigens is suggested by several lines of evidence. Studies of teleost fishes, including two other species of damselfish (family Pomacentridae), have demonstrated acute initial rejection and accelerated second set rejection of allografts in all species tested (18, 19). In addition, preliminary studies of the immune responses of bicolor damselfish have demonstrated the ability of leukocytes in these fish to recognize and respond to alloantigens and to exhibit appropriate cytotoxic activity (20). Thus, it is likely that all donor cells were destroyed within several weeks of injection. This hypothesis is also supported by the observation of granuloma formation at several injection sites where tumors developed. However, experiments were not conducted to directly evaluate the fate of injected materials in host fish.

Tumor growth as a result of allografts of neoplastic cells to a nonimmunocompromised, noninbred host has been documented in a few animal systems, such as the infectious venereal sarcoma of dogs (21) and the reticulum cell sarcoma of Syrian hamsters (22). It should be noted, however, that the canine sarcoma stimulates a conspicuous immune response that results in spontaneous regression of tumors in most dogs and that laboratory populations of Syrian hamsters typically lack the ability to reject normal skin or tumor allografts, apparently due to a lack of polymorphism in the major histocompatibility complex in these populations (23). In addition, several types of ascites tumors, such as the Ehrlich and Yoshida ascites, and their corresponding solid tumors have been adapted after many serial passages to growth in outbred or unrelated animals. These tumors are typically characterized by extremely rapid cell proliferation that results in the death of animals receiving injections within about 6 to 25 days (24).

Many of the tumors produced in these experiments were found in close association with peripheral nerves that were histologically similar to those seen within spontaneously occurring neurofibromas. These nerves were in various stages of transition leading to plexiform neurofibromas. This observation suggests that the Schwann cells of host fish were transformed by some agent present in the tumor homogenate.

Numerous examples of chemical carcinogenesis have been reported in fishes (25). However, none of the tumors tested has been shown to be transplanta ble in the absence of the carcinogenic chemicals (26). Our experience with maintaining these fish in the laboratory over periods of several years has indicated that the water sources and general environmental conditions in our laboratory are not, by themselves, carcinogenic. Thus, it seems unlikely that the transmitted tumors could have been produced solely by chemical carcinogens. Several types of viruses have been implicated in tumors in fishes. The best evidence for viral oncogenesis in a fish is in the lymphosarcoma of pike and muskellunge that has been associated with a retrovirus (27, 28).

These carcinogenic mechanisms need not be mutually exclusive. Intact donor cells might be necessary for some period after injection to allow time for production of sufficient transforming material, whether virions, growth factors, or cellular DNA, to induce the necessary transformation steps in host cells (29). Additional experiments using cell-free tumor preparations and concentrated cell fractions for inoculations will be required to determine if this agent requires the presence of intact cells from the donor, at least for a brief time following inoculation, in order to be carcinogenic.

Studies of many of the oncogenic retroviruses, such as RSV, MSV, and MLV, have shown that tumors develop more rapidly in younger individuals exposed to the viruses (30). In addition, the competence of the host's immune system often limits the growth rate and degree of spread of tumors, such that very young animals are much more susceptible to developing widely disseminated, rapidly growing tumors following injections of retroviruses such as MLV (31). Similarly, individuals that are immunosuppressed as a result of stress or other infections may show a dramatically increased incidence of tumors (32). However, a different relationship between age and tumor susceptibility is reported in systems such as murine mammary tumor virus where increases in hormone production following sexual maturity facilitate the development of tumors (33). We have not investigated the mechanisms responsible for the difference in tumor latency between juvenile and adult damselfish. Changes in immunocompetence with age may be one factor in this difference. However, the juvenile fish used in these studies should not be regarded as immunologically equivalent to newborn animals. The coincidence between long tumor latency times and sexual maturity may also indicate that changes in level of hormones or growth factors alter the susceptibility of these fish to tumor development.

Localization of the experimentally induced tumors to the sites of injection as seen in these fish is a common finding when tumors are induced experimentally via exposure to a virus.
Several factors, such as the route, dosage, and strain of the virus injected, can determine the degree of localization of tumors (34). Increased concentrations and repeated passaging of RSV and MSV have been shown to produce more widely distributed tumors with more rapid growth rates (30, 33). Injection of MSV s.c. and i.m. has been shown to produce localized lesions, while other injection routes produced widely disseminated sarcomas (30). Such localization has been reported even in the case of agents such as RSV that are found to be widely circulating in the blood of animals given s.c. or i.m. injections (34). The formation of tumors at injection sites in some of these experimental systems has been shown to be facilitated by tissue damage caused by the inoculation procedure and by the subsequent process of wound healing (34).

There is no evidence to suggest a genetic etiology for DNF. However, a genetic predisposition or susceptibility to peripheral nerve sheath tumors may be present in certain bicolor damselfish populations or in the species as a whole. A study of Schwann cell tumors in coho salmon suggested that these tumors may have been produced, at least partially, by genetic factors (35). If a virus is responsible for DNF, this would suggest that transformation was occurring either via introduction of a viral oncogene or by the alteration in expression of a cellular gene (by insertional mutagenesis or activity of a transacting gene such as the tat gene of HTLV-1). Many of the retroviral oncogenes thus far identified have been found to have cellular counterparts involved in human cancers (16). In addition, the recently produced transgenic mouse model of NF suggests that several retroviral gene sequences from HTLV-1 may be responsible for inducing development of neurofibromas in these animals (8). Thus, identification of either a viral or a cellular oncogene in DNF might have direct applicability to the identification of the gene(s) and gene products responsible for NF in humans.

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Transmissibility of a Neurofibromatosis-like Disease in Bicolor Damselfish

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