ANTIFREEZE PROTEIN IN WINTER FLOUNDER, *Pleuronectes americanus*, GILL EPITHELIAL CELLS ISOLATED AND GROWN IN CULTURE

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Antifreeze protein in winter flounder, *Pleuronectes americanus*, gill epithelial cells isolated and grown in culture.

by

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Abstract

Antifreeze proteins (AFPs) have been found in the blood of many teleost species and have the ability to bind to ice crystals and inhibit their growth. Type I AFP was later discovered in several body tissues of the winter flounder (*Pleuronectes americanus*) including the skin, scales and gills. In order to further our understanding of these proteins that are not produced in the liver, epithelial cells of the winter flounder gill were isolated and maintained in culture to look for the presence of skin type I AFP. The present study is the first that describes the isolation and culture of gill epithelial cells from winter flounder. The isolation procedure used was based in part on the gill cell isolation method developed by Pärt *et al.* [Pärt, P., Norrgren, L., Bergström, E. & Sjöberg, P.(1993) *J. Exp. Biol.* **175**, 219 - 232]. The presence of type I AFP in the cells was determined using immuno-histochemistry. The results indicate that epithelial cells stained positive for winter flounder type I AFP antisera against liver, skin or a recombinant form of type I AFP whereas cells stained with sea raven type II AFP antiserum showed no reaction. The distribution pattern of AFP seen in these cells suggests the AFP is located intracellularly. The AFP produced within these cells is believed to be the skin type I AFP and is thought to react with the liver and recombinant antisera due to the close similarity between these AFPs. In short term culture, individual cells were of three predominate shapes. Round cells were 8.3 ± 2.6 μm in diameter, crescent cells were 19.8 ± 5.2 μm x 10.4 ± 2.9 μm and elongated cells were 21.5 ± 5.0 μm x 6.8 ± 1.7 μm. Scanning electron microscopy (SEM) showed individual cells with an elevated nuclear region with a low and somewhat ruffled outer cytoplasmic area. Dishes that contained a relatively high number of isolated gill cells supported the formation of a confluent monolayer of cells. Scanning electron micrographs of confluent cells 8 days in culture show a highly flattened apical surface with no distinguishing characteristics. The cells attached to culture dishes and were capable of forming confluent monolayers of cells similar to pavement cells seen in other gill epithelial cultures. However, SEM revealed that they do not contain microridges typically seen in pavement cells isolated from the sea bass and rainbow trout. The use of the fluorescent stain DASPEI showed these cells to be mitochondria-rich, a characteristic of gill epithelial chloride cells. Preparation of cultures such as these provide a means of examining the mechanisms involved in skin type I AFP production, regulation and also how these proteins function in gill epithelia.

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List of Abbreviations

AFP	antifreeze protein
AFGP	antifreeze glycoprotein
EDTA	ethylenediaminetetraacetic acid
DHS	dialyzed horse serum
FBS	Fetal bovine serum
MOPS	3-[N-morpholino]propane-sulfonic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) N, N, N', N'- tetraacetic acid
PBS	phosphate buffered saline
DAB	diaminobenzidine
DASPEI	2-(4-dimethylamino)styryl)-N-ethylpyridium iodide
DASPMI	2-(4-dimethylamino)styryl)-N-methylpyridium iodide
DMF	dimethylformamide
SEM	scanning electron microscopy

1. Introduction

1.1 General introduction

Many fish species live in polar and subpolar waters with temperatures near the freezing point of seawater. Resistance to freezing by fish that inhabit these waters has been attributed to the presence of unusual serum proteins and glycoproteins (Raymond & DeVries, 1977). These antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) lower the blood serum freezing temperature noncolligatively without affecting the melting point (DeVries, 1971). AFPs are also produced in some species of insects, centipedes, intertidal molluscs, and plants that can survive freeze-thawing (Carpenter & Hanson, 1992). AFPs isolated from fish and insects are extremely effective at inhibiting ice recrystalization during warming, even at concentrations as low as 1 μg/ml (Carpenter & Hanson, 1992). Although several types of AFGPs isolated from different fish species all have similar chemical structures, there are a variety of AFPs that have been broadly classified into three major groups: AFP types I, II and III (Davies & Hew, 1990).

1.2 Type I AFP

This class of AFPs has been studied from two distinct phylogenetic families and is the most widely characterized. Type I AFP was first isolated from the northern winter flounder (*Pleuronectes americanus*) of the righteye flounder family by Duman and DeVries (1974). Other type I AFPs that have been studied come from the cottid family including the shorthorn, arctic and grubby sculpins (Yang *et al.*, 1988).

Type I AFPs are a family of seven independently active alanine rich (60 mol %) compounds ranging in molecular weight from 3.3 kDa to 4.5 kDa (Yang *et al.*, 1988). They are α-helices that contain at least three 11 amino acid repeats (Thr-X_2-Asp/Asn-X_7), where X is predominantly Ala (Baardsnes *et al.*, 1999).

Chou (1992) reports that the type I AFP molecule from winter flounder contains four threonine residues (i.e. Thr2, Thr13, Thr24 and Thr35) that are nearly aligned parallel to the α-helix axis and separated successively by ~ 1.61 nm, a spacing that is very close to the ~ 1.66 nm spacing along the [01$\bar{1}$2] direction in the ice lattice. Chou (1992) proposed that this spacing suggests the AFP molecule binds to the ice lattice in a zipper-like fashion through hydrogen bonding of the four threonine side-chains to the oxygen atoms in the [01$\bar{1}$2] direction in the ice lattice, thus inhibiting the formation of ice pyramidal planes which subsequently depresses the freezing point. However, studies by Baardsnes *et al.*, (1999) propose a new ice-binding face for type I AFP. Experiments that substitute Leu for Ala in positions 19 and 20 of the α-helix demonstrated wild-type antifreeze activity, whereas a substitution in position 21 confers only 10% antifreeze activity as compared to the wild-type and a substitution in position 17 eliminates all antifreeze activity. This suggests that Thr13, Ala17 and Ala21, and the equivalent residues at 11 amino acid intervals along the α-helix, make up the ice-binding face for type I AFP and is responsible for its antifreeze activity.

The preceding information describes a type I AFP that is produced in the liver and secreted in the blood. We now know that species such as the winter flounder produce a non-liver type I AFP in many tissues throughout the animal (see section **1.7 Skin and liver type I AFP of the winter flounder**).

1.3 Type II AFP

Type II AFP was first isolated from the sea raven (*Hemitripterus americanus*) by Ng *et al.* (1986) and has also been found in Atlantic herring and smelt (Ewart & Fletcher, 1990). This group of AFPs range in molecular weight from 14.5 kDa to 16 kDa (Slaughter *et al.*, 1981) and share a high protein sequence identity (Ewart *et al.*, 1996). Type II AFPs contain a significant amount of half-cystine and average amounts of alanine (Ng *et al.*,1986). They have a β structure with five disulfide bridges, have little helical content (Ng *et al.*,1986) and do not have a repeating unit (Ewart & Fletcher, 1990).

The mechanism by which type II AFP binds to ice is not yet understood and the surface that forms the ice-binding site is not known (Ewart *et al.*, 1996). Ng and colleagues (Ng *et al.*, 1986) suggest that the reverse turns in the sea raven AFP might be responsible for its antifreeze activity. The polar nature of the turns and their general appearance on the surface of the protein may be involved in forming hydrogen bonds with water.

Ewart *et al.*, (1992) demonstrated that the cDNA for a type II AFP isolated from smelt is very similar to a family of Ca^{2+}-dependent lectins and that Ca^{2+} is required for type II AFP to be an effective antifreeze.

1.4 Type III AFP

Type III AFP was first isolated from the Newfoundland ocean pout (*Macrozoarces americanus*) (Hew *et al.*, 1984) and can be divided into five different groups based on ion exchange chromotography (Hew *et al.*, 1988). This family of AFPs range in molecular weight from 6 kDa to 7 kDa and have no distinguishing secondary structural features (Hew *et al.*,

1988). This type of AFP lacks repeating ice-binding residues. It is a compact protein with an angular structure and is composed of numerous short, imperfect β-strands and one α-helical turn (Jia *et al.*, 1996).

Ice binding is believed to occur by means of a flat amphipathic ice-binding site where five hydrogen-bonding atoms match two ranks of oxygen molecules on the $\{10\bar{1}0\}$ ice prism plane resulting in high ice-binding affinity and specificity (Jia *et al.*, 1996).

1.5 AFGP

DeVries and Wohlschlag (1969) were the first to isolate AFGP from notothenиid fishes of the Antarctic Ocean. These glycoproteins were later found in northern cod by Fletcher *et al.* (1982). There are 8 types of AFGPs; types I to V all have molecular weights greater than 10.5 kDa and possess strong antifreeze properties (Rubinsky, *et al.* 1990). The smaller types VI to VIII (7.9 kDa, 3.5 kDa, and 2.6 kDa respectively) however, have relatively weaker antifreeze activities (Rubinsky, *et al.* 1990). AFGPs are characterized by having tripeptide repeats $(Ala\text{-}Ala\text{-}Thr)_n$ with a disaccharide bound to threonine (DeVries, 1971).

The polypeptide backbone of the AFGP molecule is thought to form a polyproline II-like left-handed helical structure with three residues per turn (Davies & Hew, 1990). The disaccharides in the molecules are believed to be arranged in a planar conformation with their hydrophillic groups exposed to the aqueous solvent. The similarity between this model and that of the amphiphilic α-helix of type I AFP may explain the functional similarity of separately evolved antifreezes (Davies & Hew, 1990).

1.6 Significance of AFP and potential uses.

Apart from an interest in AFPs and AFGPs in terms of their importance in cold water fish, these proteins may also prove to be important for other purposes. For instance, because of the properties of AFPs and AFGPs in cold water protection, they may be useful in providing protection to mammalian tissues during cold preservation.

If membranes could be made to be less leaky (leakage due to passive diffusion) during periods of cold storage to balance the cold-induced reduction in metabolism, then membrane polarity would remain intact resulting in the prevention of Ca^{2+} induced cytotoxicity. Many researchers have investigated the potential use of AFPs and AFGPs in combating the problem of membrane ion leakage during cold storage. Early studies with AFPs dealt with its effect on ice crystallization and recrystallization (Raymond & DeVries, 1977; Schneppenhiem & Theede, 1982; Carpenter & Hanson, 1992; Hanson *et al.*, 1993; Jia *et al.*, 1996). However, other studies have been conducted to determine if AFPs (or AFGPs) directly interact with the exterior of cell membranes (Hays *et al.*, 1996; Rubinsky *et al.*, 1990, 1992).

Studies by Rubinsky *et al.* (1992) provided evidence that AFPs have the ability to block Ca^{2+} and K^{-} fluxes across the membranes of porcine granulosa cells, an effect they attributed to a direct interaction between the AFPs and the membrane ion channels. In addition, experiments by Hays *et al.* (1996) show that AFGPs prevent leakage of a trapped marker from liposomes as they are cooled and warmed through the membranes transition temperature. This suggests that AFPs may interact directly with the lipid phase of the membrane. Taken together, these two studies demonstrate that AFPs and AFGPs interact directly with components of the membrane and raise the possibility they have a role in cellular

activity.

Other investigators have experimented with AFPs and AFGPs effect on tissue and organ viability after cold storage and cryopreservation. Rubinsky *et al.* (1990) conducted a study on AFGPs from Antarctic nototheniid fishes and their ability to protect pig oocytes from hypothermic damage. The results show that a combination of the different AFGPs is able to protect the structural integrity of the oocyte oolemma and inhibits ion leakage at 4°C. However, a study by Wang *et al.* (1994) using AFGPs from Antarctic nototheniid fishes to protect rat cardiac explants found that AFGPs failed to protect the cardiac explant from hypothermic damage.

Several studies have also been conducted using AFPs and AFGPs for protection against cell and tissue damage during cryopreservation. Examination of pig oocytes incubated in cryogenic temperatures using types I, II, and III AFP and AFGP by Arav *et al.* (1993) demonstrated that the oolemma of the oocytes was protected by each of the four proteins. Similar results were not seen however when KG-1a cells, a human myelogenous leukemia cell line from bone marrow, were cryopreserved in differing concentrations of dimethylsulfoxide and type I AFP (Hansen *et al.*, 1993). Cell recovery was not improved with the AFP and high concentrations of the protein caused a large reduction in cell viability. Similarly, Wang *et al.* (1994) found that the addition of AFGP to rat cardiac explant during cryopreservation increased damage regardless of the AFGP concentration. It would appear from these results that AFPs and AFGPs may provide protection against cold storage, but in most cases, may be harmful if used at cryogenic temperatures.

The commercial use of antifreeze proteins in both aquaculture and agriculture has also

been examined by several researchers in an attempt to extend the temperature range in which a product may be produced or grown. Wang *et al.* (1994) microinjected Ocean pout type III AFP genes into the oocytes of goldfish and found that goldfish that tested positive for the AFP transgene were significantly more cold tolerant than control goldfish when challenged with low temperatures. Such experiments have led Hew & Fletcher (1997) to experiment with incorporating the winter flounder AFP gene into the genome of the Atlantic salmon in an attempt to develop a freeze tolerant transgenic salmon that will extend northward the range of Atlantic salmon farming in Atlantic Canada. Although the AFP expression in these salmon are low (~ 50μg/ml) and is insufficient to confer freeze tolerance, attempts are being made to boost AFP levels by increasing gene dosage and by developing a better AFP (Hew & Fletcher, 1997).

Kenward *et al.* (1993) worked on transferring a type I AFP gene from winter flounder into tobacco plants. Although western blot analysis of the plant tissue did not confirm the presence of AFP, they found that transgenic plants did contain AFP mRNA. They did however find that the levels of AFP were detected in transgenic plants only after exposure to cold. Western blot analysis shows the presence of a protein that co-migrated with the winter flounder proAFP and that cross reacted with type I AFP antisera. Later experiments by Wallis *et al.* (1997) showed that expression of a synthetic AFP gene in potato plants (*Solanum tuberosum* - russet burbank) can reduce electrolyte leakage from leaves during periods of freezing. Electrolyte release analysis of transgenic leaves shows a correlation between the degree of freezing tolerance and the amount of transgenic protein expression. This work was the first to produce a transgenic plant that showed a tolerance to freezing.

Although some studies have not found AFPs and AFGPs to be beneficial during cold storage, several researchers have shown these proteins to be advantageous when cold preserving tissues. The potential medical and commercial importance of these unusual proteins necessitates understanding how they are regulated *in vivo* and how they may be produced *in vitro*.

1.7 Skin and liver type I AFP in the winter flounder

Perhaps the most characterized and studied AFPs are type I AFPs from the winter flounder (*P. americanus*). Investigation of the plasma of the Newfoundland winter flounder by Fourney *et al.* (1984), showed the presence of seven active type I AFP components; five of 3.3 kDa and two of approximately 4.5 kDa.

The first studies indicating that the AFP found in the blood of the winter flounder is produced in the liver were conducted by Hew & Yip (1976). AFP is now known to be produced in the liver as large precursor molecules, 82 amino acids long (Davies *et al.*, 1982; Pickett *et al.*, 1984). The presequences of this AFP precursor are thought to be removed cotranslationally while the prosequence is cleaved off soon after secretion of the AFP precursor into the blood (Hew *et al.*, 1986).

Fletcher *et al.* (1985) compared the annual cycles of plasma AFP in three different populations of winter flounder: Conception Bay, Newfoundland; Passamaquoddy Bay, New Brunswick; and Shinnecock Bay, Long Island, New York. AFP levels in blood plasma correlate closely with seasonal changes in water temperature, however the differences in AFP levels between populations did not correlate with sea water temperature (Fletcher *et al.*,

1985). The investigators found that the differences between populations correlated with the regional differences in the time of water temperature increase. They hypothesized that plasma AFP production is a genetically determined population characteristic determined by the earliest date that AFP is needed for survival (Fletcher *et al.*, 1985).

Later studies indicate that AFP gene expression in winter flounder is also hormonally regulated (Fletcher *et al.*, 1989). Transcription of AFP gene was shown to be repressed by growth hormone during the summer. However, during the fall, growth hormone release by the central nervous system is suppressed primarily by photoperiod through the hypothalmohypophysial axis, thus AFP gene transcription is increased enabling the winter flounder to tolerate icy waters (Fletcher *et al.*, 1989). Fourney *et al.* (1984) demonstrated that hypophysectomizing fish during the summer months resulted in a significant increase in AFP mRNA levels in the liver, up to as much as a 40 fold increase as reported by Gong *et al.* (1995). Furthermore, Fletcher *et al.* (1989) demonstrated that injection of growth hormone into hypophysectomized winter flounder inhibited the increase of AFP mRNA transcription as seen by Fourney *et al.*, (1984) by blocking AFP mRNA transcription.

The liver is an important site of AFP synthesis in many species of fish (Hew & Yip, 1976). However, Gong *et al.* (1992) found that type I and III AFP mRNA were also found in several non-liver tissues of the winter flounder and ocean pout, respectively, notably in the skin, scales and gills. No such non-liver tissues of the sea raven were found to contain type III AFP mRNA. These findings indicate that AFP production is confined to the liver in the sea raven, whereas AFP synthesis occurs in several body tissues in winter flounder and ocean pout.

Experiments by Pickett *et al.* (1983) have shown that liver AFP mRNA ranges from 0.5% of the total liver mRNA during the coldest winter months to only 0.0007% during the summer. More recently, Gong *et al.* (1995) have shown using northern blot analysis, that liver AFP mRNA undergoes a several hundred fold difference between winter and summer months, whereas AFP mRNAs from the gills and kidney only display a moderate 5 to 10 fold difference.

A great deal is known about the regulation of the liver AFPs, however much less information is available on the mechanisms involved in the synthesis and regulation of the non-liver (skin) AFPs. As previously stated, hypophysectomizied fish showed over a 40 fold increase in AFP mRNA levels, however non-liver AFP mRNA levels in these fish did not significantly increase (Gong *et al.*, 1995). This suggests that there is a different mechanism responsible for the modest increase in non-liver AFP mRNA.

Although it is apparent that photoperiod is not a major contributor to the regulation of non-liver AFP mRNA, evidence suggests temperature may be in part responsible for the seasonal cycles of non-liver AFP mRNA (Gong *et al.*, 1995). Using an *in vivo* run-on analysis, Vaisius *et al.*, (1989) did not find any evidence that low temperatures stimulate non-liver AFP gene expression. Thus, the effect that temperature has on non liver AFP mRNA levels appears to be posttranscriptional at the level of RNA stability with low temperature increasing the half-life of AFP mRNA (Lindquist & Peterson, 1990).

Not only are liver and non-liver AFPs differently regulated, it has also been shown that there are amino acid differences between type I AFP from the liver and non-liver (skin) tissues of the winter flounder. Gong *et al.* (1996) found that the skin type I AFP contained 11

similar amino acid repeats that are found in liver type I AFP. However the mature skin AFP lacked the signal peptide that is found in liver type I AFP. This implies that the skin type I AFP plays an intracellular role, unlike the role of liver type I AFP which is secreted into the blood.

Immuno-histochemical studies of the gill of the winter flounder by Murray *et al.* (1997) indicate that the pavement cell is responsible for the appearance of skin type I AFP in the gill epithelia of the winter flounder. Therefore, the focus of this project was to isolate and culture pavement cells from the gill of the winter flounder and to determine if this cell type is responsible for, or contributes to, the observation of AFP in the gill. Pavement cells from other teleost fish have successfully been isolated and grown in culture including the rainbow trout (*Oncorhynchus mykiss*) (Pärt *et al.*, 1993) and the sea bass (*Dicentrarchus labarax*) (Avella *et al.*, 1994). A procedure was developed to isolate and culture the pavement cells of the winter flounder gill epithelia based partially on the procedure developed by Pärt *et al.* (1993). A technique to isolate and maintain cells that are known to produce the skin type I AFP could provide us with more insight on the regulatory mechanisms responsible for skin AFP gene expression as well as its possible role within these tissues.

1.8 Winter flounder life history

As the work in the present study focuses on AFP type I of the winter flounder, a brief description is given of the habitat and features of this fish.

The winter flounder is a righteye flounder belonging to the family Pleuronectidae (Class Osteichthyes, Order Pleuronectiformes) (Scott & Scott, 1988). It is an inshore species

inhabiting soft muddy to hard bottoms in shallow water. It is commonly found in depths of 1.8 - 3.6 m (Scott & Scott, 1988), but has also been found in depths of 143 m by McCracken (1954). Winter flounder inhabit the inshore coastal areas of Atlantic Canada and northeastern United States throughout the year (Fletcher *et al.*, 1985). This species undergoes what is considered regular onshore-offshore migration, moving offshore in the winter and inshore in the summer (Scott & Scott, 1988).

Generally, winter flounder are larger in southern regions as compared to more northern regions at a given age, but there is much variability in regional growth rates. Inshore fish are usually smaller than those found offshore. The average length for mature males is about 20 cm and mature females about 25 cm when they are 3-4 years old (Scott & Scott, 1988) but some winter flounder can be up to 46 cm in length at 13 years (Kennedy & Steele, 1971).

Winter flounder are sight feeders that do most of their feeding during daytime hours. They consume more food in the summer months than in winter. They feed on a variety of bottom organisms depending on their habitat, including polychaete worms, bivalves, gastropods, crustaceans, planktonic crustaceans, and bottom invertebrates (Frame, 1974). Winter flounder have also been shown to feed heavily on caplin eggs in Conception Bay, Newfoundland (Kennedy & Steele, 1971).

Winter flounder are important prey for harbor, harp and grey seals (Fischer & Mackenzie, 1955) and are common prey for ospreys in the summer. Young flounder are fed upon by blue herons and cormorants (Tyler, 1971) and also by monkfish, dogfish and sea raven during the winter while in deeper waters (Dickie & McCracken, 1955).

1.9 Winter flounder gill anatomy and physiology

The gross anatomy of the gill of the winter flounder is quite similar to many other species of teleost fish. Located beneath the bony operculum on either side of the fish are four branchial or gill arches which extend from either side of the pharynx. Each gill arch contains two rows of gill filaments which are oriented parallel to each other and perpendicular to the gill arch (see Materials & Methods, Figure 1). Support for the gill filaments is provided by a cartilaginous rod which extends from the gill arch into the gill filament (Conte, 1969). The filament is further divided into gill lamellae which are very thin structures that are the main site of gas exchange. These lamellae extend above and below the axial plane of the filament and are referred to as respiratory or secondary lamellae (Conte, 1969).

The fish gill, unlike many other types of epithelia, performs several functions including gas exchange, acid/base regulation, ion regulation, and nitrogenous waste excretion (Pärt and Bergström, 1995). Gill epithelium is composed of several cell types including chloride (mitochondria-rich) cells, mucous (goblet) cells, accessory cells, and respiratory (pavement) cells (Laurent & Dunel, 1980).

Pavement cells comprise as much as 90-95% of the total surface area of the gill and typically display a complex system of microridges and microvilli on their apical surface (Pärt *et al.*, 1993). However, Avella and Ehrenfeld (1997) found that pavement cells cultured with fetal bovine serum instead of fish serum had irregularities in the typically organized apical membrane and that some cells lacked any organization with an almost smooth surface. The primary role of pavement cells was believed to be gas exchange, however Avella & Ehrenfeld (1997) have found evidence that pavement cells are also involved in ion regulation, a process

previously believed to be confined to only chloride cells.

1.10 Objectives

As previously stated, the gill epithelia of the winter flounder have been shown to possess type I AFP, and it is believed that the pavement cell is responsible for its production in the gill (Murray *et al.*, 1997). The aim of this project was to isolate and establish primary cultures from cells isolated from the gill of the winter flounder and to determine if these cells possess the skin type I AFP. Among the different cell types of the gill, only the pavement cell has been the most successfully isolated and maintained in culture.

Once primary cultures were established, the purpose of the project was twofold. I wanted to: 1) determine if intracellular skin type I AFP is present in these cells and, 2) use scanning electron microscopy to determine if the cells isolated resemble pavement cells.

2. Materials and Methods

2.1 Animals

Winter flounder (*Pleuronectes americanus*) were obtained from the Ocean Sciences Center of Memorial University of Newfoundland, Logy Bay. They were collected from Conception Bay, Newfoundland and then kept in open circuit tanks with natural photoperiod and ambient temperature seawater (-1°C to 15°C) from Logy Bay.

2.2 Solutions

All solutions were prepared using tissue-culture quality chemicals.

2.2.1 Cell isolation

Fish saline: 175 mM NaCl, 2.7 mM KCl, 0.64 mM $MgCl_2$, 2.74 mM $CaCl_2$, 2.22 mM glucose, 3.0 mM Tris. The final pH of all solutions was adjusted to 7.8. Ca^{2+}/Mg^{2+} free fish saline: 175 mM NaCl, 2.7 mM KCl, 2.22 mM glucose, 3.0 mM Tris. Trypsinizing solution: 0.05% trypsin (Sigma, St. Louis, MO), 0.02% EDTA (Sigma, St. Louis, MO) in Ca^{2+}-Mg^{2+}-free fish saline. Stopping solution: 10% dialyzed horse serum (DHS) (GIBCO BRL, Burlington, ON) in fish saline. Washing solution: 2% DHS in fish saline.

2.2.2 Cell culture

Culture medium: Leibovitz L15 (GIBCO BRL, Burlington, ON) supplemented with 10% fetal bovine serum (FBS) (GIBCO BRL, Burlington, ON), 20 mM NaCl, 100 units/ml penstrep (penicillin and streptomycin), and 100 μg/ml gentamycin. All antibiotics were purchased from GIBCO BRL (Burlington, ON).

2.2.3 Antisera

Table 1 lists the primary antisera used in type I AFP detection experiments. The primary antisera WF, Recom, Skin and WF2 were produced in rabbits to one of several sources of type I AFPs while the SR2 antiserum was produced in rabbit to type II AFP from sea raven liver. All primary antisera were supplied by Dr. C. Hew (Department of Biochemistry, The Hospital for Sick Children, Toronto, ON., Canada) except for the Recom primary antiserum which was supplied by Dr. P. Davies (Department of Biochemistry, Queens University, Kingston, ON, Canada).

WF and WF2 antisera were both produced to liver type I AFP of winter flounder at different times from different fish. The Recom antiserum was produced from type I AFP which was manufactured in an *Escherichia coli* expression system. The Skin antiserum was produced against winter flounder type I AFP isolated from skin. The SR2 antiserum was produced against type II AFP isolated from the sea raven. The secondary antisera was goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) purchased from Sigma (St. Louis, MO). Antisera solutions were made and applied as indicated below.

2.2.4 AFP detection using antisera

MEMFA fixative: 10% 3-[N-Morpholino]propane-sulfonic acid (MOPS) (Sigma, St. Louis, MO), 0.4% EGTA, 0.1% $MgSO_4$, 10% formaldehyde in distilled water. Phosphate buffered saline (PBS): 70 mM NaCl, 3 mM KCl, 10.1mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4. Primary antisera solutions: 0.2% of each primary antisera (listed in Table 1), 5% goat serum (Sigma, St. Louis, MO) in PBS. Secondary antiserum solution: 0.2% secondary antiserum (goat anti-rabbit IgG-HRP), 5% goat serum in PBS. Diaminobenzidine (DAB)

Table 1: List of antisera used in antifreeze detection experiments.

Antibody Used	Source
WF[1]	polyclonal antisera to winter flounder liver type I AFP produced in rabbit.
Recom[2]	polyclonal antisera to a recombinant type I AFP (produced in *E. coli*) produced in rabbit.
Skin[1]	polyclonal antisera to winter flounder skin type I AFP produced in rabbit.
SR2[1]	polyclonal antisera to sea raven liver type I AFP produced in rabbit.
WF2[1]	polyclonal antisera to winter flounder liver type I AFP produced in rabbit.

[1] Antisera were obtained from Dr. C. Hew, Department of Biochemistry. The Hospital for Sick Children. Toronto, ON., Canada.

[2] Antiserum was obtained from Dr. P. Davies, Department of Biochemistry, Queens University, Kingston, ON. Canada.

solution: 1x solution prepared from ImmunoPure® Metal Enhanced DAB Substrate Kit (Pierce, Rockford, IL).

2.2.5 DASPEI staining

A 5 mM stock solution of 2-(4-dimethylamino)styryl)-N-ethylpyridium iodide (DASPEI) (Molecular Probes, Inc., Eugene, OR) was made using dimethylformamide (DMF). The DASPEI staining solution was then made by diluting the stock solution to 10 μM with fish saline.

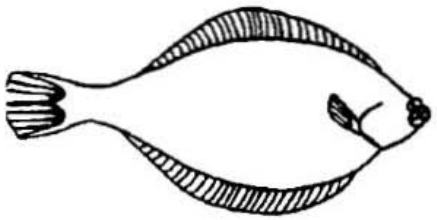
2.2.6 Scanning electron microscopy

Karnovsky's fixative and cacodylate buffer were both obtained from Lisa Lee (Biology Department, Memorial University) and Howard Gladney (Faculty of Medicine, Memorial University). Karnovsky's fixative: 4% paraformaldehyde solution in 0.2M sodium cacodylate buffer, prepared as described by Karnovsky (1965). Sodium cacodylate buffer: 0.2 M sodium cacodylate, pH 7.4.

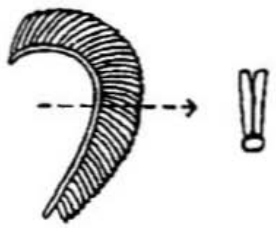
2.3 Cell isolation

Winter flounder were killed by a blow to the head followed by cervical severance. A schematic diagram of the cell isolation procedure is provided in Figure 1. The gill arches were dissected and rinsed for approximately 1, 5, and 15 min each in fish saline at room temperature. Cells were isolated from four gill arches at a time. The gill filaments were excised from the gill arches and rinsed three times for 15 min each and thereafter twice for 5 min each in 5 ml changes of Ca^{2+}/Mg^{2+} free fish saline. The Ca^{2+}/Mg^{2+} free fish saline was removed, replaced by 2 ml of trypsinizing solution and the gill filaments were incubated at

Isolation of winter flounder gill epithelial cells

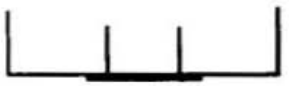


⇨ 8 gill arches
- 4 pairs
- 2 pairs on each side



⇨2 rows of gill filaments per arch
⇦ cross section of gill arch

Gill filaments are
excised and trypsinated
to remove epithelial cells



⇨ Isolated cells are placed in culture dishes and incubated at 4°C
- short term cultures
- long term cultures

Figure 1: Schematic diagram of the procedure for the isolation of winter flounder gill epithelial cells. Refer to Materials & Methods for cell isolation details.

room temperature on a shaker (American Optical Corp., model 02156) at approximately 50 agitations per minute for 15 min. The cell suspension was then removed and placed in 2 ml of stopping solution. New trypsin solution was added to the remaining filament and the above procedure was repeated. The cell suspensions from both digestions were then centrifuged at 1800 rpm for 10 min. The cell pellets were pooled and washed twice with washing solution by resuspension and centrifugation. The final cell pellet was resuspended in 10 ml of fish saline, placed in culture dishes with glass coverslip bottoms and incubated at 4°C. All cultures were prepared within 24 hr of gill dissection.

2.4 Culture conditions

After 24 hr incubation in fish saline, the cells were rinsed three times with fish saline and then incubated in culture medium. The cultures were maintained at 4°C at atmospheric pCO_2.

2.5 AFP detection

Cell cultures used for AFP detection were all between 1 and 8 days old. Cells were fixed with MEMFA for 30 min at 4°C, washed three times for 5 min each with 0.1% Tween 20 (BDH Inc., Toronto, ON) in PBS and then treated with 20% goat serum in PBS for 15 min. Following treatment with goat serum, one of the primary antisera was added for 30 min. The cells were washed three times for 5 min each with 0.1% Tween 20 in PBS. The secondary antisera solution was then added to the dishes for 30 min and the cells were washed three times for 5 min each with 0.1% Tween 20 in PBS. DAB solution was added to the

cells for 5 min in a fumehood and the cells washed three times for 5 min each with 0.1% Tween 20 in PBS. The coverslips containing the cells were removed from the culture dishes, and mounted on glass slides using Gel/Mount™ (Biomeda Corp., Foster City, CA). Unless otherwise specified, all solutions were added to the cells at room temperature on a Vibrax VXR S1 shaker (IKA Labortechnik, West Germany).

2.6 DASPEI staining

Cell cultures used for DASPEI staining were all between 1 and 9 days old. Cells were washed three times with fish saline and the DASPEI stain was added to the cells for 40 min at room temperature in dark conditions. The cells were then rinsed with fish saline and observed in the culture dish.

2.7 Microscopy

After isolation or culture the cells were visualized at 400X magnification under phase contrast, brightfield and fluorescence optics using a Zeiss IM-35 microscope.

2.8 Scanning electron microscopy

Scanning electron microscopy (SEM) was performed at the Electron Microscopy Facility in the Department of Biology, Memorial University with the help of Ms. C. Emerson or Ms. L. Lee. One and 8 day cultures were examined using SEM. The coverslips with attached cells were rinsed three times with fish saline and fixed for 1 hr at 4°C in Karnovsky's fixative. The cells were rinsed and stored in cacodylate buffer. The samples were dehydrated

in a graded series of ethanol and critical point dried from liquid carbon dioxide in a Polaron E 3000 critical point drying apparatus. The coverslips containing the cells were attached to aluminum stubs with either silver paint or conductive double back tape, gold coated in an Edwards Model 150A sputter coater, and examined with a Hitachi S570 scanning electron microscope operated at an accelerating voltage of 20 kV.

3. Results

3.1 Establishment of primary cultures

Short term cultures of single isolated cells from gill epithelia were prepared to determine if these cells possessed the type I AFP. Once short term cultures were established, an attempt was made to grow these cells in long term culture.

3.1.1 Short term cultures

Once the gill filaments were trypsinated and the resulting cell suspension incubated for 24 hr, the culture dishes contained a thin film of mucous containing several gill epithelial cell types including numerous red blood cells similar to those seen by Pärt and Bergström (1995). Some of the mucous remained from the isolation procedure, however mucous cells that remained after the isolation, may have also produced mucous during the 24 hr incubation. In order to observe the cells that would attach to the bottom of the glass coverslip of the culture dishes, the mucous film had to be removed along with the other cells in the dish that do not adhere to the coverslips. The dishes were rinsed three times with fish saline to remove non-attached cells and mucous, leaving only cells attached to the glass coverslips. Rinsing the dishes undoubtedly removed some attached cells as well since considerable rinsing is needed to remove the mucous. This led to, in some cases, dishes that had very few cells attached to the dish. Typically, the less mucous in the dish after the 24 hr incubation, the more cells remained attached to the dish, presumably due to less rinsing.

Although the cells that adhered to the coverslips were of various shapes, there appeared to be three predominant shapes that were observed in the cultures, these being round, crescent, and elongated shapes (Figure 2). Cells described as being round were the

Figure 2: Phase contrast micrographs of winter flounder gill epithelial cells in 24 hr culture. The large arrows show crescent and elongated shaped cells (Figures 2A and 2B respectively) while small arrows show round shaped cells. A single red blood cell can also be seen (rbc arrow) (scale bar = 20μm).

smallest of the cell shapes, with a central nuclear region and little lateral cytoplasm (small arrows - Figure 2A & 2B). These round cells had an average diameter of 8.3 ± 2.6 μm (n = 15). Crescent shaped cells had the nucleus located on one side of the cell, with the other three sides surrounded by cytoplasm (large arrow - Figure 2A). They had an average length of 19.8 ± 5.2 μm and a width of 10.4 ± 2.9 μm (n = 15). Cells described as being elongated appeared long and thin or short and thick with the nuclear region in the middle of the cell and cytoplasm at both ends of the cell (large arrow - Figure 2B). On average, elongated cells were 21.5 μm ± 5.0 in length and a width of 6.8 ± 1.7 μm (n = 15). A representative sketch of each described cell shape is included with Table 2. All measurements were taken from cells that had been fixed, treated with the WF antiserum and mounted on slides.

3.1.2 Long term cultures

Dishes that contained a relatively high density of cells (approximately 35 cells per 62500 μm^2) after 24 hr of incubation, were then re-incubated with culture medium. After three to four days in culture medium, small scattered clusters of cells were seen. After 38 days in culture medium, aggregations of confluent cells could be seen throughout the dish (Figure 3). Figure 3A shows an aggregation of about 10 cells (large arrow) with other smaller groups of cells (medium arrow) and single cells (small arrows). A much larger aggregation of confluent cells can be seen in Figure 3B. There are however, some areas where the cells have not become confluent (i.e., not grown together) as indicated by arrows in Figure 3B. Cells at confluence had the appearance of a 'pavement', similar to cultured rainbow trout epithelial cells seen by Pärt *et al.* (1993). Individual cells no longer had crescent and elongated shapes previously described (small arrows - Figure 2 & 3A) but

Table 2: Description and average size (μm) of three common cell shapes of single winter flounder gill epithelial cells that have been fixed, treated with WF antiserum and mounted on slides. Five cells were observed and measured from cultures made on May 28/97, November 4/97, and January 30/98 for each cell shape (n = 15).

Cell shape	Average size*	Cell description
round 	8.3 ± 2.6 μm	small, round compact cells with little lateral cytoplasm.
crescent 	19.8 ± 5.2 μm x 10.4 ± 2.9 μm	crescent shaped cell, nucleus located to one side of the cell surrounded by cytoplasm on three sides.
elongated 	21.5 ± 5.0 μm x 6.8 ± 1.7μm	long, thin to short, thick cells with the nucleus in the middle of cell with cytoplasm at both ends of the cell. Some cells appear 'bow tie' shaped.

* 5 cells were measured in each of 3 cultures (n = 15).

Figure 3: Phase contrast micrographs of winter flounder gill epithelial cells in culture for 39 days showing aggregations of confluent cells. The large arrow in Figure 3A shows an aggregation of about 10 cells, the medium arrow shows a smaller group of cells and the small arrows show single cells. In Figure 3B, the arrows point to spaces between aggregate cells (scale bar = 20μm)

appeared polygonal and more rounded in appearance.

It should be noted however, only a few dishes containing the single, isolated cells produced confluent aggregations of cells. Only those dishes with a relatively high density of single cells after the initial cell isolation supported the development of confluent cells.

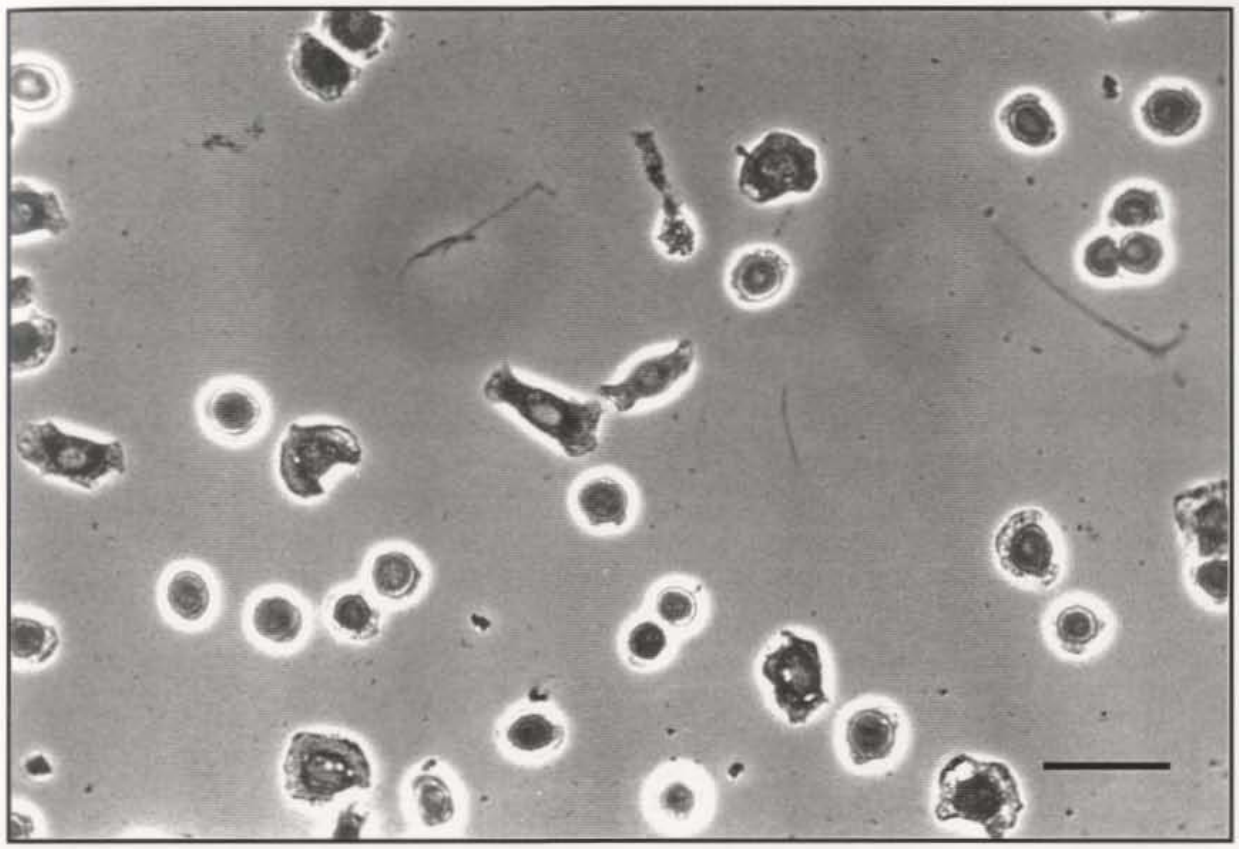
3.2 AFP detection using antisera

To determine if these cells possessed the type I AFP, a number of different antisera were used (Table 1). A combination of both phase and brightfield microscopy was used to ascertain which antiserum tested positive for the presence of its corresponding antigen.

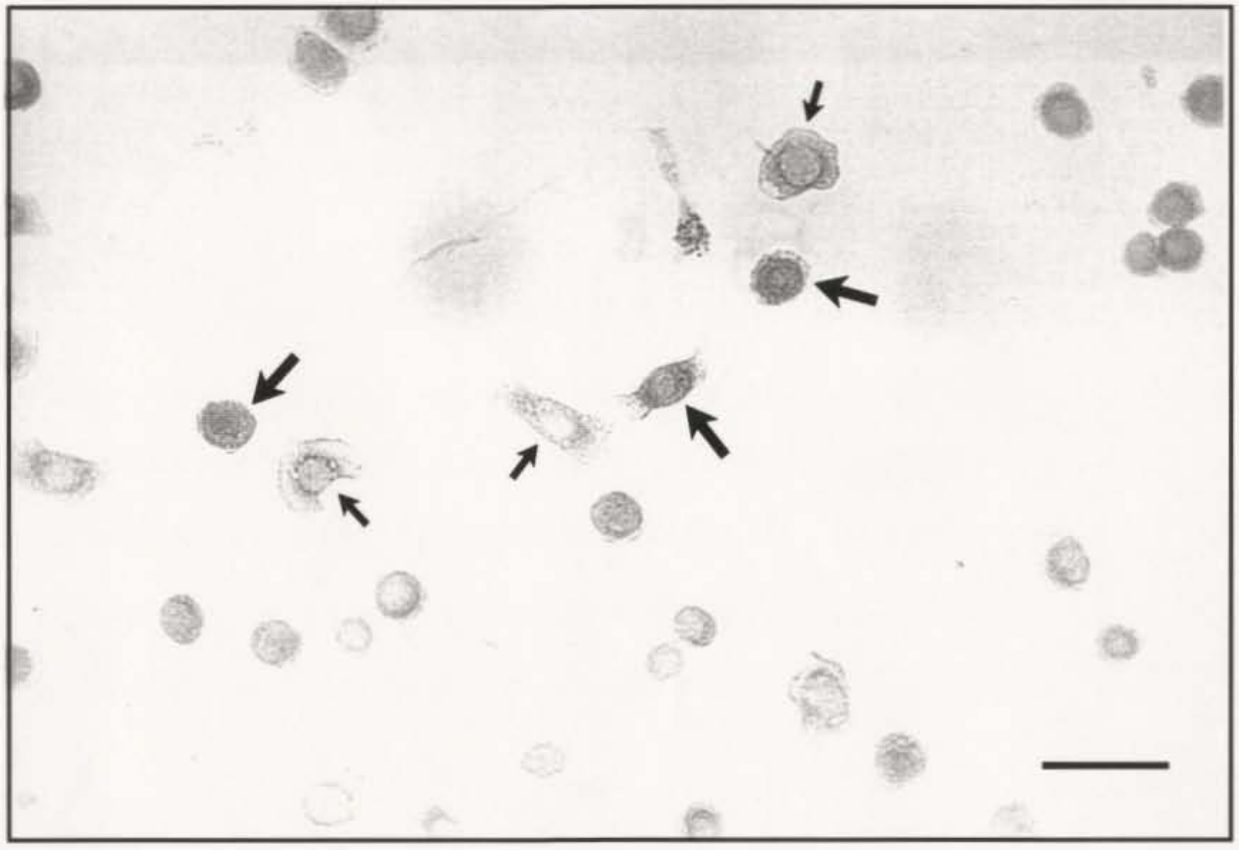
Cells observed using phase optics can be seen regardless of whether the cells have been stained or not. Only those cells that showed a dark brown-black precipitate using brightfield optics were considered positive. The precipitate results from the cleavage of the DAB substrate by the HRP enzyme conjugated to the secondary antibody. Cells that were not visible (no DAB-HRP reaction product present) were considered negative.

Figure 4 shows phase and brightfield (A and B respectively) micrographs of cultured cells that have been treated with the WF antiserum. All cells that can be seen in the phase micrograph (Figure 4A) have stained positive using the WF antiserum and are visible under brightfield optics (Figure 4B). The staining appears to be concentrated mostly around or associated with the central nuclear region of the cell as seen in some round and elongated shaped cells (large arrows) whereas in other cells, the staining appears to have a more generalized distribution throughout the cell with a higher density located around the periphery of the central nuclear region (small arrows). Differences in WF staining within

Figure 4: Phase contrast (A) and brightfield (B) micrographs of winter flounder gill epithelial cells in 24 hr culture after fixation with MEMFA and treatment with WF antiserum and DAB. The large arrows in Figure 4B point to cells that have the WF antiserum concentrated in the central nuclear region while the small arrows show cells that have the antiserum appearing mostly in the periphery of the central nuclear region (scale bar = 25μm).



A



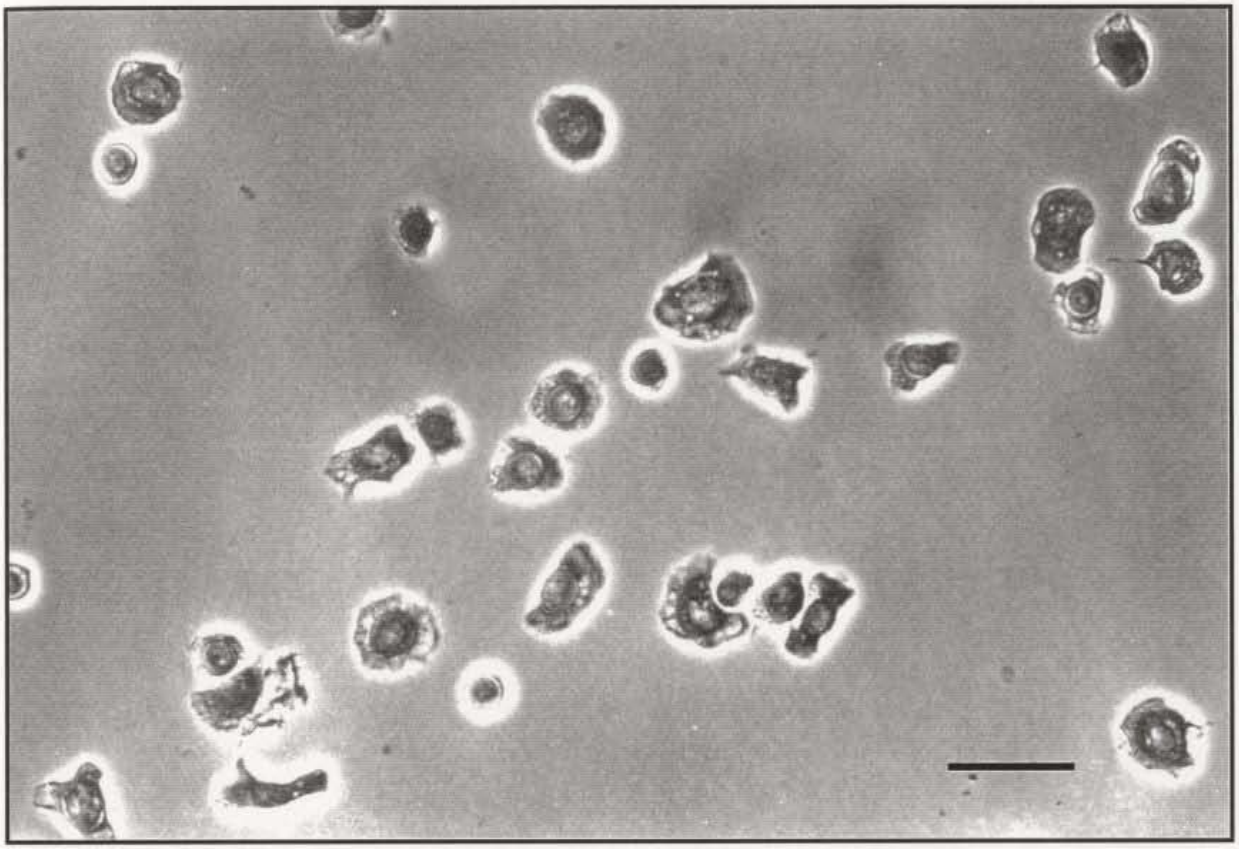
B

cultures is most likely due to differences in cell cytoplasm density (thickness). These results demonstrate that the cells isolated from the gill epithelium test positive for the WF antigen (liver type I AFP).

Phase and brightfield (A and B respectively) micrographs of cultured cells that have been treated with the Recom antiserum (Figure 5). All cells that can be seen in the phase micrograph (Figure 5A) have stained positive using the Recom antiserum and are visible under brightfield optics (Figure 5B). Figure 5B shows that the cells have taken up the Recom antiserum in much the same manner as with the WF antiserum. The Recom antiserum appears to be mostly concentrated in the periphery of the central nuclear region of the cells as can be seen in some round, crescent and elongated shaped cells (small arrows 1, 2, and 3 respectively) while other cells have a more generalized staining throughout the cell (large arrows). Staining in these areas with the Recom antiserum also appears to be more dense when compared to cells stained using the WF antiserum, although this may not be the case since the staining densities have not been measured quantitatively. These results demonstrate that the cells isolated from the gill epithelium test positive for the Recom antigen (liver type I AFP).

Phase and brightfield (A and B respectively) micrographs of cultured cells that have been treated with the Skin antiserum (Figure 6). It appears that all cells that can be seen in the phase micrograph (Figure 6A) have stained using the Skin antiserum and are visible under brightfield optics (Figure 6B). Figure 6B shows that the cells have stained in dense concentrated areas, namely between the central nuclear region of the cell and the thin peripheral cytoplasm as can be seen in round and elongated shaped cells (arrows - Figure

Figure 5: Phase contrast (A) and brightfield (B) micrographs of winter flounder gill epithelial cells in 24 hr culture after fixation with MEMFA and treatment with Recom antiserum and DAB. Small arrows 1, 2, and 3 in Figure 5B show cells (round, crescent and elongated shaped respectively) that have the Recom antiserum concentrated mostly in the periphery of the central nuclear region. Large arrows show cells that have a more generalized staining (scale bar = 25μm).



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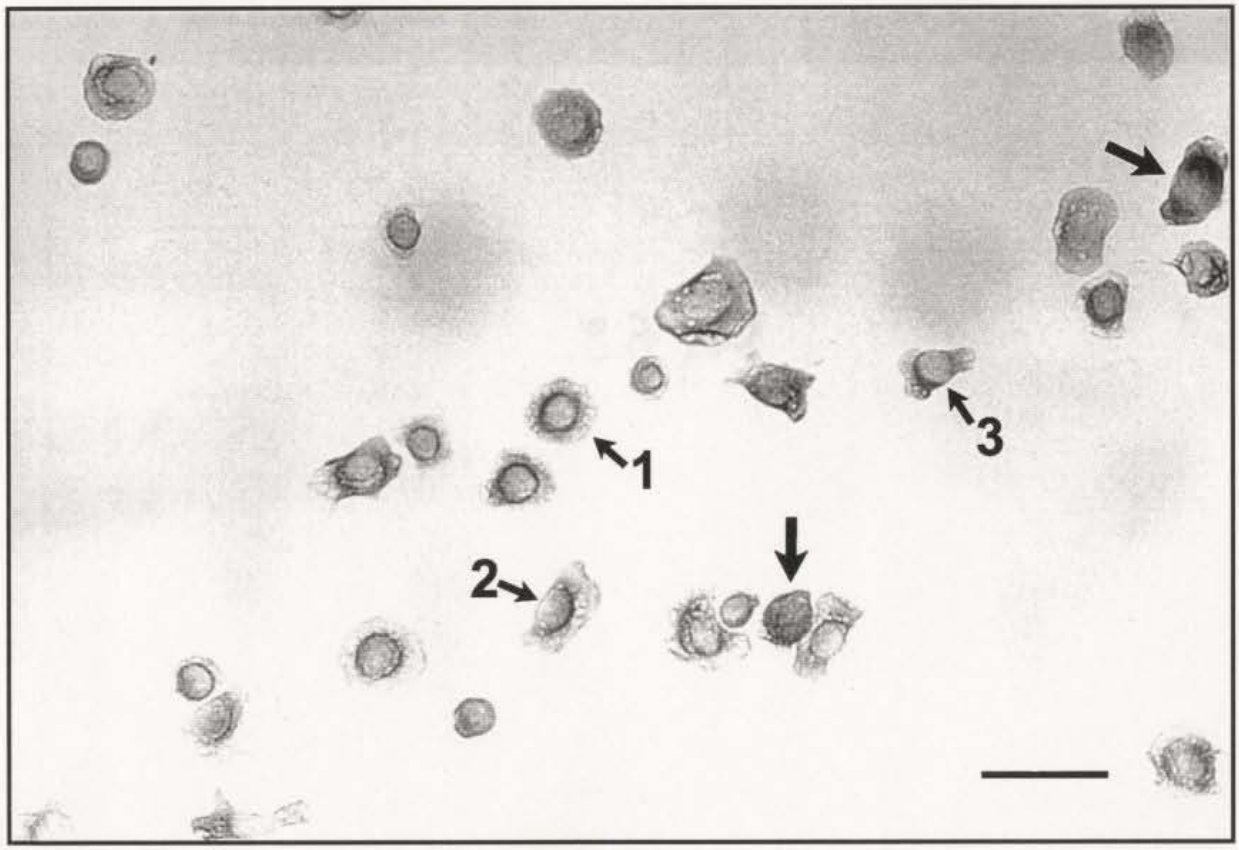
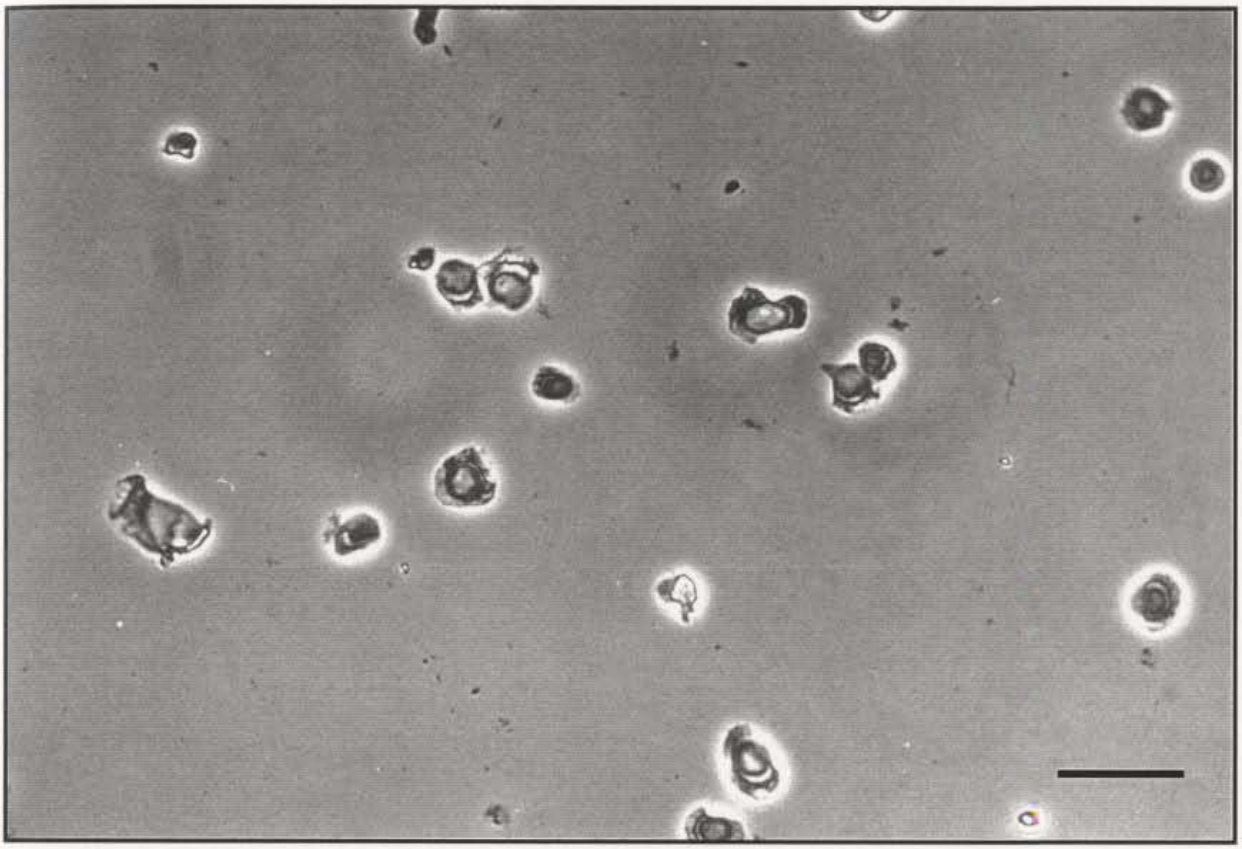


B

Figure 6: Phase contrast (A) and brightfield (B) micrographs of winter flounder gill epithelial cells in 24 hr culture after fixation with MEMFA and treatment with Skin antiserum and DAB. Arrows in Figure 6B show dense areas of Skin antiserum located mainly between the central nuclear region and the thin peripheral cytoplasm (scale bar = 25μm).



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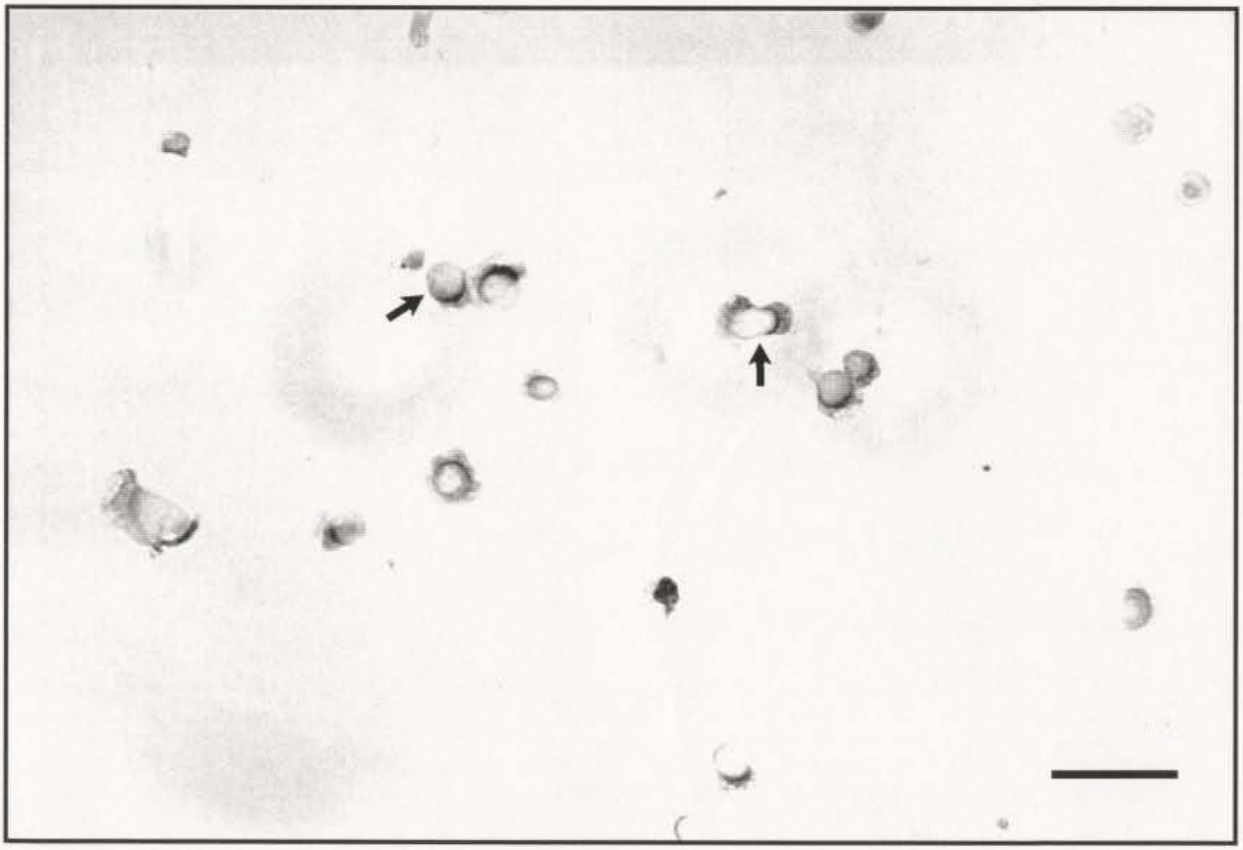


B

6B). There also appears to be less antigen located in the central nuclear region of these cells compared to that seen in previous brightfield micrographs. These results demonstrate that the cells isolated from the gill epithelium test positive for the presence of the Skin antigen (skin type I AFP).

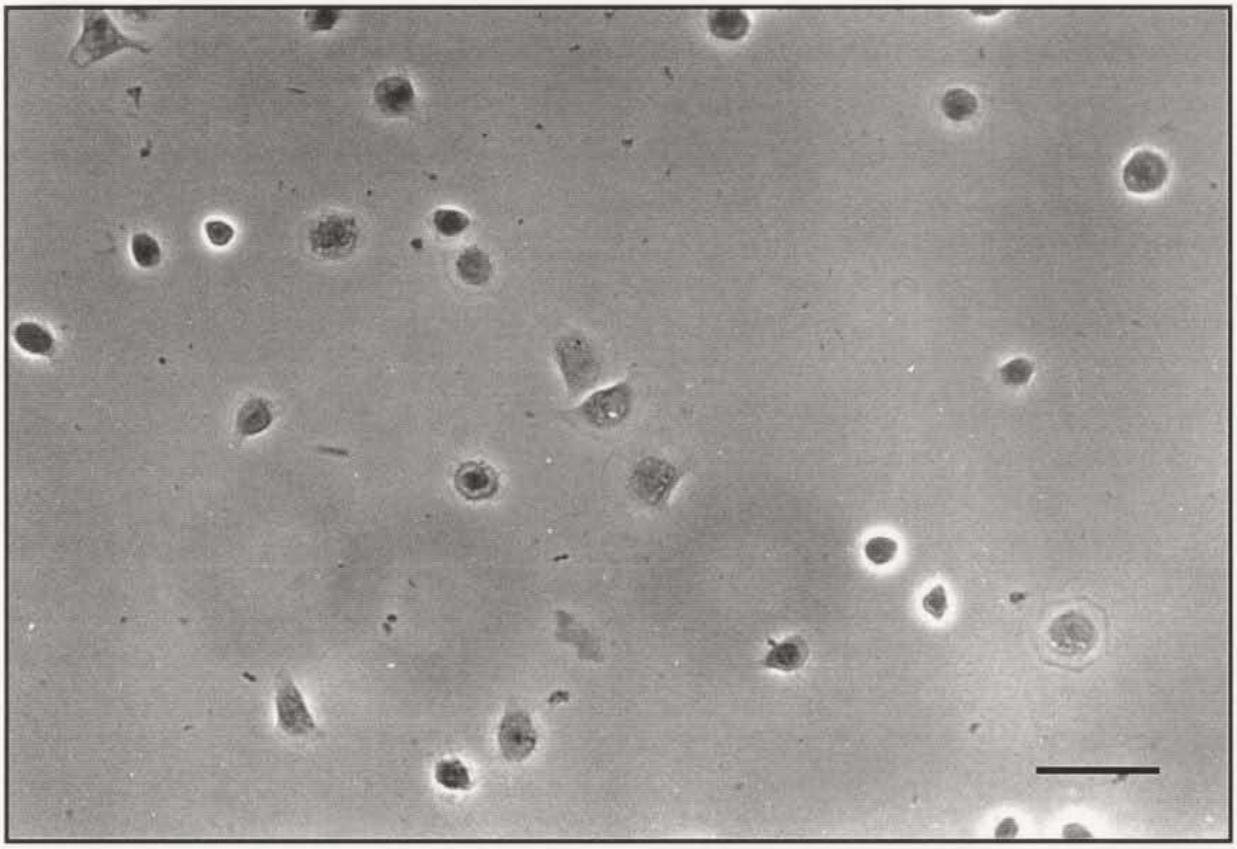
Phase and brightfield (A and B respectively) micrographs of cultured cells that have been treated with the WF2 antiserum (Figure 7). In contrast to the cells in Figures 4B through 6B, the cells in Figure 7 are not visible when treated with the WF2 antiserum and viewed using brightfield optics. These results demonstrate that the cells isolated from the gill epithelium test negative for the WF2 antisera (liver type I AFP).

Phase and brightfield (A and B respectively) micrographs of cultured cells that have been treated with the SR2 antiserum (Figure 8). Like the cells treated with WF2 antiserum, the cells treated with the SR2 antiserum test negative for the presence of an SR2 antigen (type II AFP).

Phase and brightfield (Figures 9A and 9B respectively) micrographs of cultured cells that have been treated the same as the cells in Figures 4 through 8 except the primary antisera was absent (negative control). Again, the cells were not visible using brightfield optics, similar to what is seen in Figures 7 and 8.

Virtually all the cells observed incubated with the WF (Figure 4), Recom (Figure 5), and Skin (Figure 6) antisera could be easily seen using brightfield microscopy indicating that these cells possess the type I AFP. Cells incubated with WF2 (Figure 7) and SR2 (Figure 8) could not be seen under brightfield optics and appeared to be similar to those cells that were not incubated with either of the antisera (Figure 9).

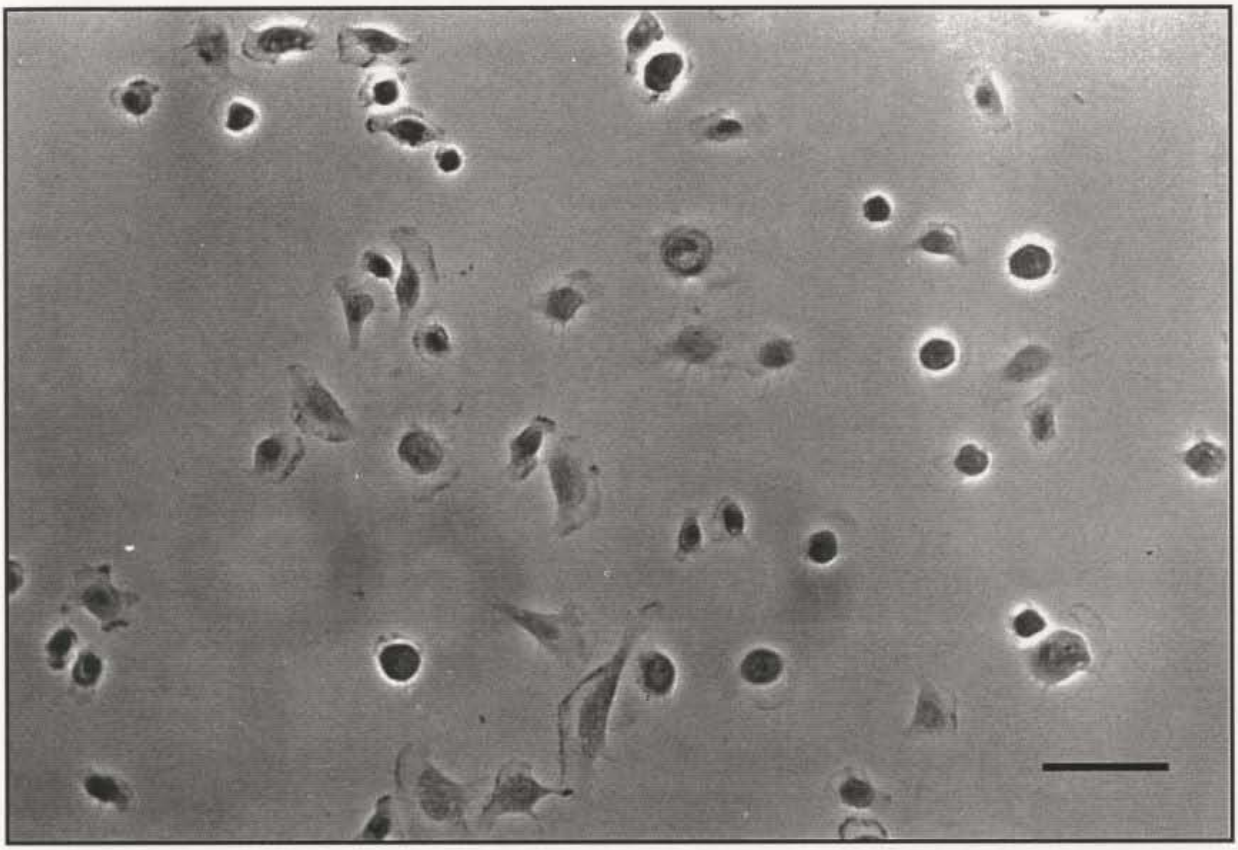
Figure 7: Phase contrast (A) and brightfield (B) micrographs of winter flounder gill epithelial cells in 24 hr culture after fixation with MEMFA and treatment with WF2 antiserum and DAB (scale bar = 25μm).



A

B

Figure 8: Phase contrast (A) and brightfield (B) micrographs of winter flounder gill epithelial cells in 24 hr culture after fixation with MEMFA and treatment with SR2 antiserum and DAB (scale bar = 25μm).



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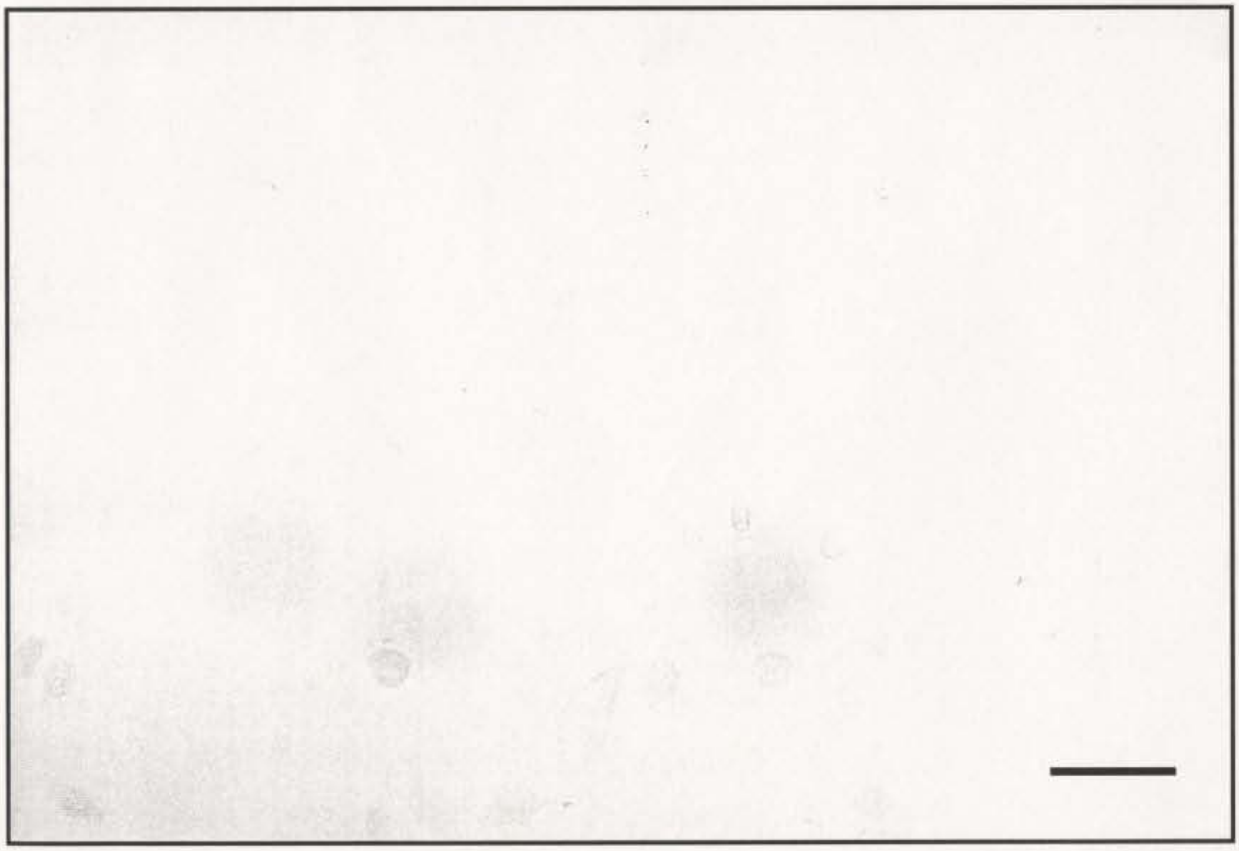
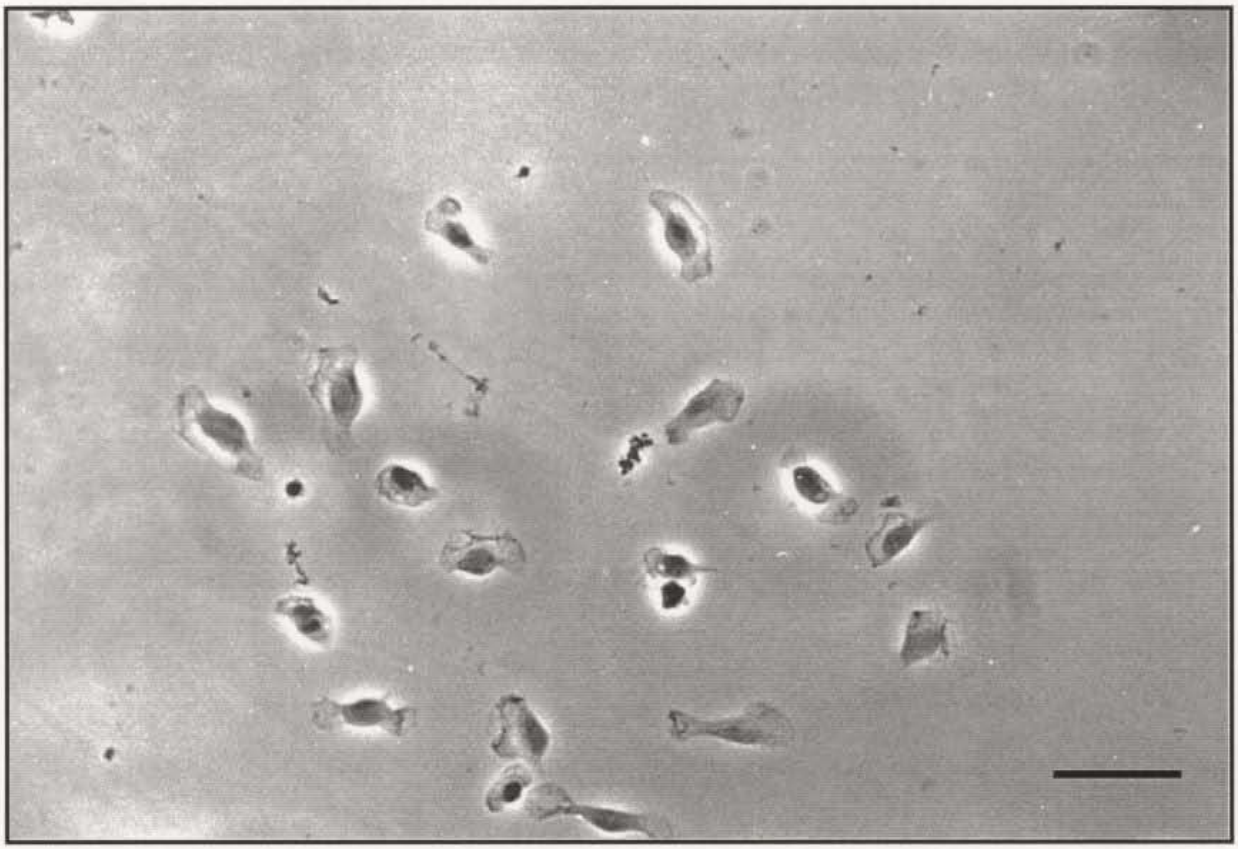
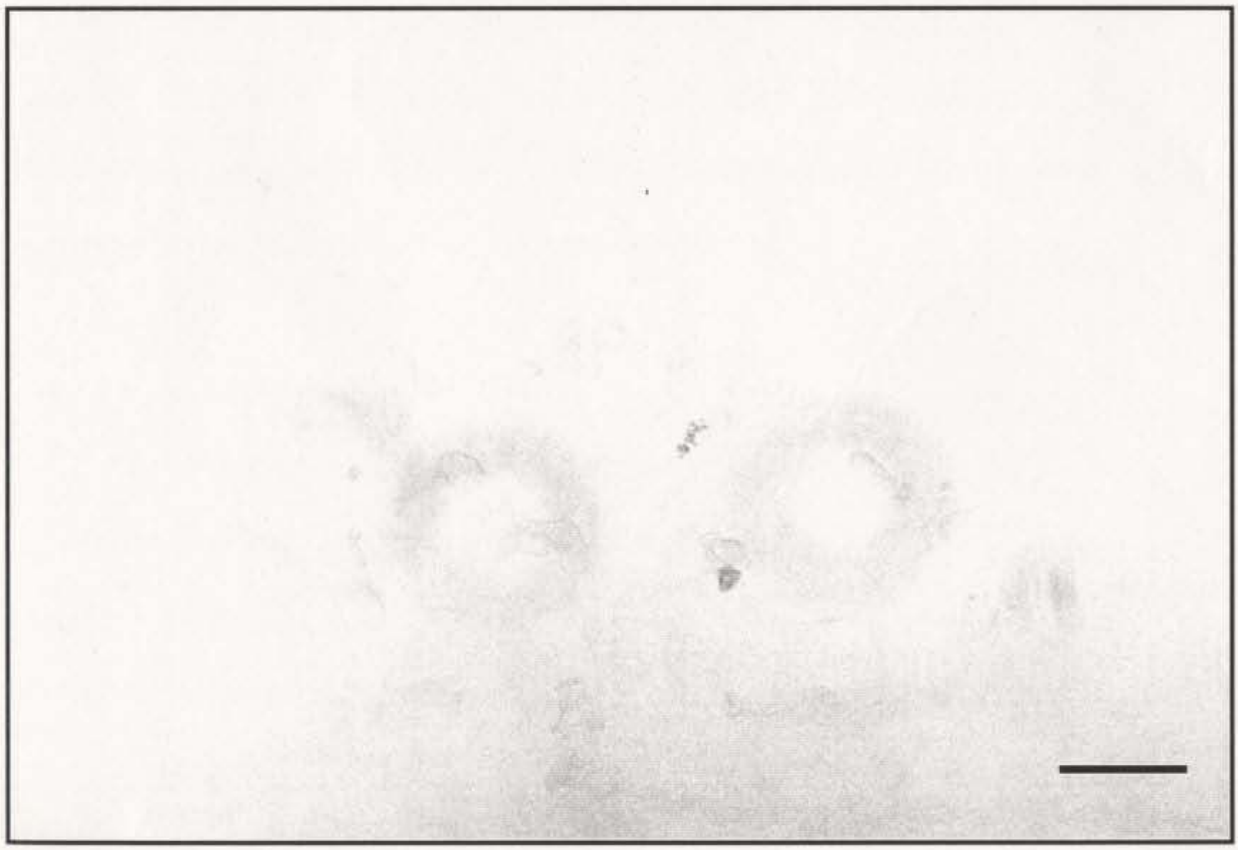


B

Figure 9: Phase contrast (A) and brightfield (B) micrographs of winter flounder gill epithelial cells in 24 hr culture after fixation with MEMFA and DAB with no antiserum added (scale bar = 25μm).



A



B

The number of slides (grouped by antisera) that have been examined for AFP and those slides that were considered positive for type I AFP are summarized in Table 3.

3.3 DASPEI Staining

A study by Marshall and Nishioka (1980) demonstrated the usefulness of using the fluorescent probe DASPEI to visualize chloride cells in the skin of the marine teleost *Gillichthys mirabilis* (longjaw mudsucker) and has since been used to identify mitochondria-rich chloride cells in many other fish species.

Phase and fluorescence (Figures 10A and 10B respectively) micrographs of 9day old winter flounder gill epithelial cells that have been treated with DASPEI. Comparison of the two micrographs indicate that each cell seen in Figure 10A has been stained with DASPEI and is visualized using fluorescence optics as seen in Figure 10B. The cells appear to have the DASPEI stain located within the central nuclear region of the cell (large arrows) with some cells having most of the stain located around the periphery of the central nuclear region (small arrows). Since the DASPEI treated cells can be visualized under fluorescence optics, it appears that these gill epithelial cells are mitochondria-rich.

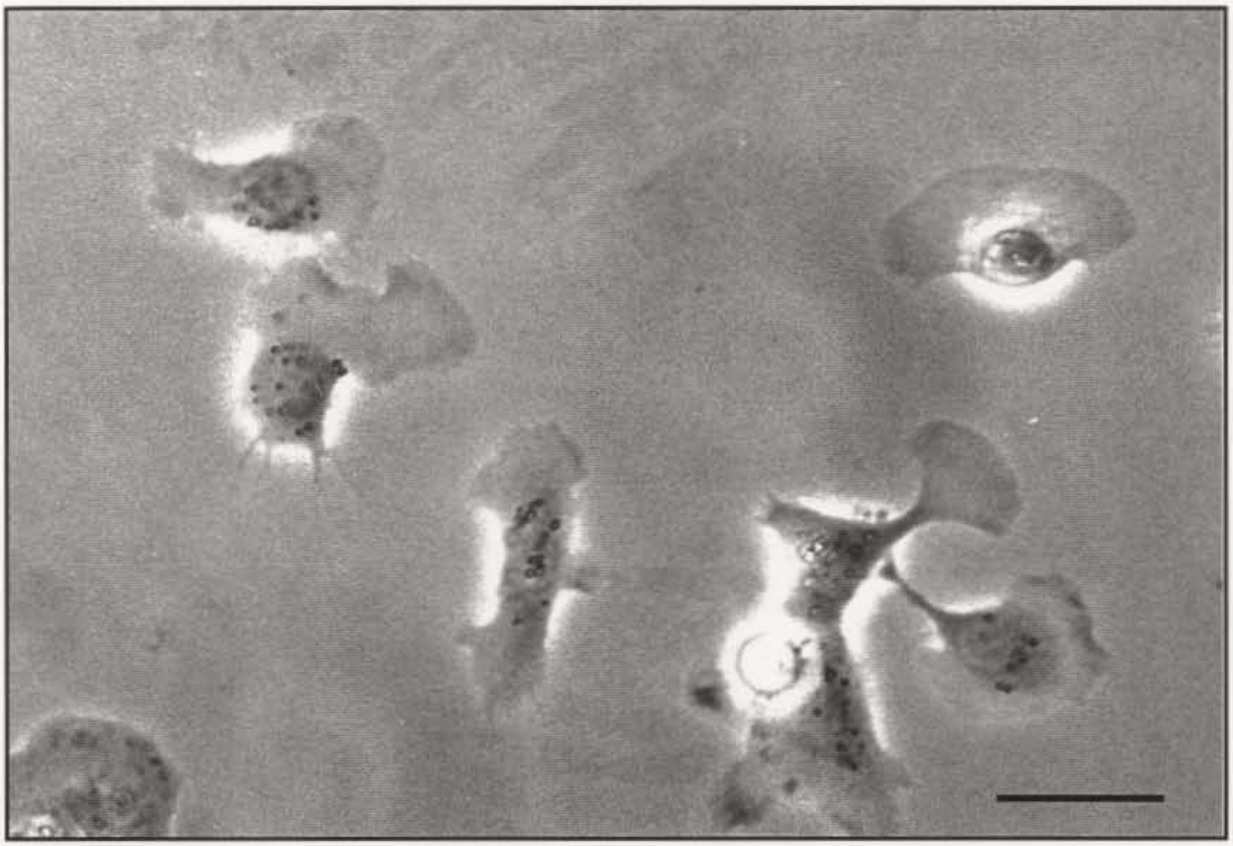
3.4 Scanning electron microscopy

The gill epithelium of the winter flounder contains several different cell types, however it was not certain which cell type remained after the isolation procedure and the removal of the mucous film. To gather more morphological information that would be useful in identifying these adherent cells, scanning electron microscopy was performed on

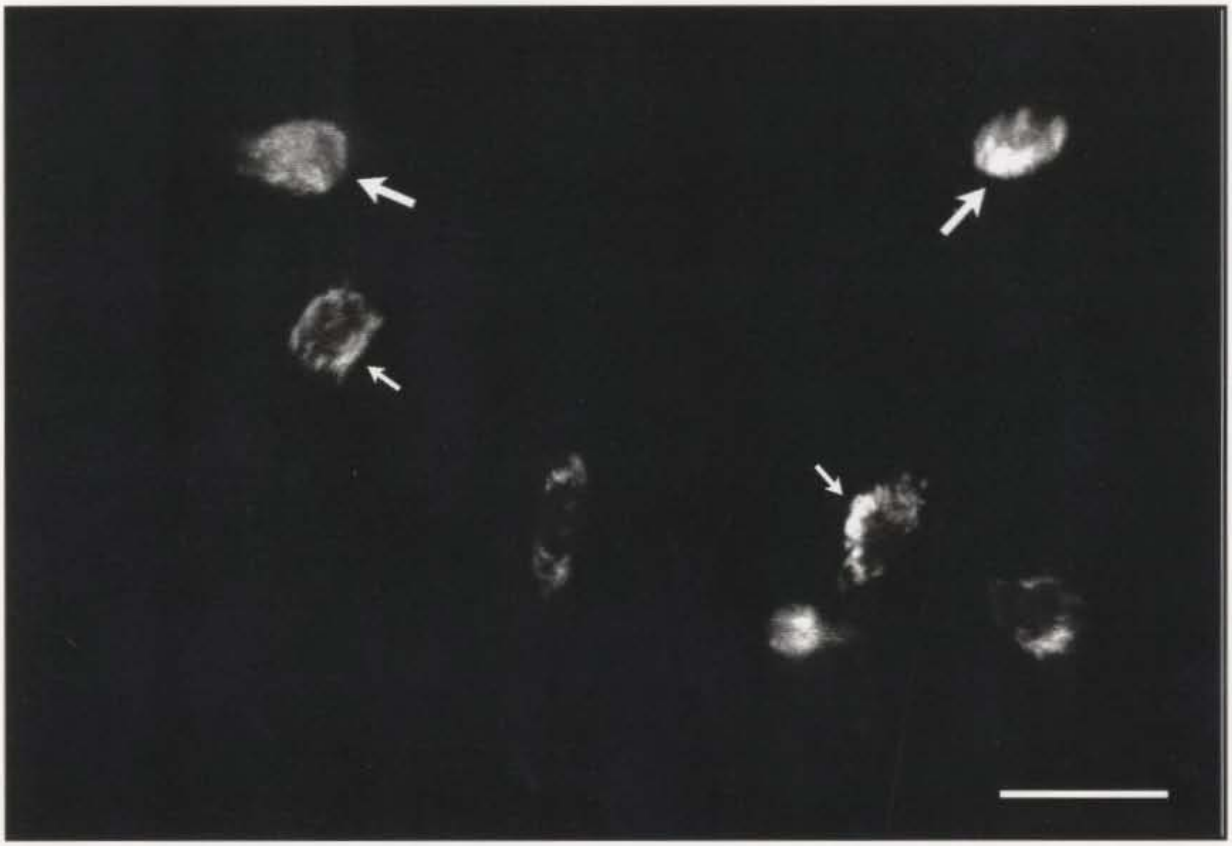
Table 3: Results of experiments using several antisera (Table 1) to detect type I AFP in gill epithelial cells isolated from winter flounder. Antisera positive for type I AFP allowed cells to be seen under brightfield microscopy whereas antisera that did not allow cells to be seen were considered negative.

	Antisera					No Antisera
	WF	Recom	Skin	SR2	WF2	
# of slides examined	19	7	2	11	4	3
# of slides considered positive	17	7	2	0	1	0
% of slides considered positive	89.5	100	100	0	25	0

Figure 10: Phase contrast (A) and fluorescence (B) micrographs of 9 day old winter flounder gill epithelial cells that have been treated with DASPEI. Large arrows in Figure 10B show cells that appear to have the DASPEI stain located within the central nuclear region of the cell whereas the small arrows show cells having most of the stain located around the periphery of the central nuclear region (scale bar = 15μm).



A



B

cells cultured for 24 hr (short term cultures), eight days, and 23 days (long term cultures) following isolation.

3.4.1 Short term cultures

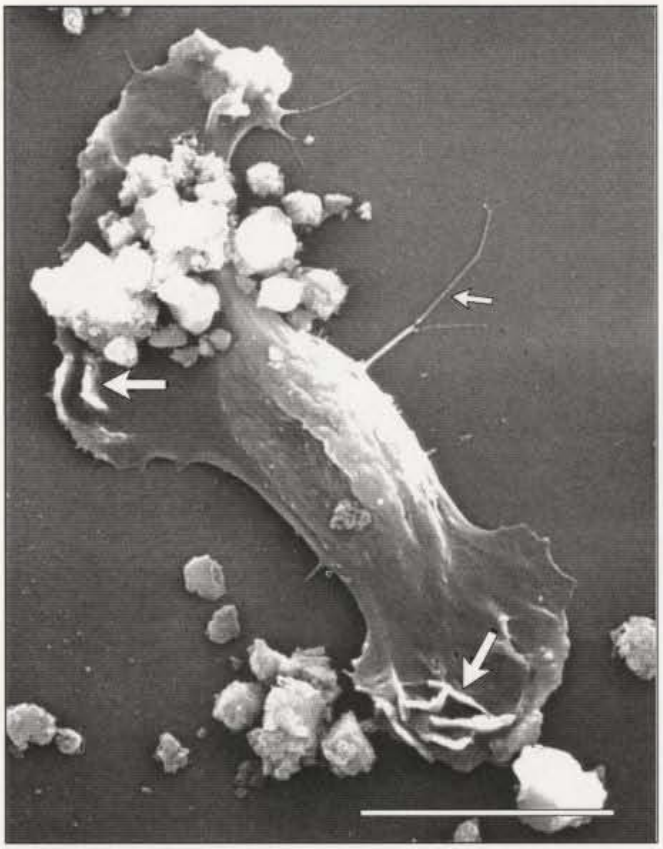
Scanning electron microscopy (SEM) of single cells show irregularly shaped cells that have a rough, elevated central nuclear region, and a flattened, smoother outer region (Figure 11). The cells appeared to be anchored to the glass coverslip via the outer, flatter regions of the membrane which contain numerous cytoplasmic protrusions that also appear to be anchored to the coverslip (small arrows). Most of the cytoplasmic region appears to be flat, however there are areas of the flattened surface that have small ruffles (large arrows). The cells seen in Figure 11 are similar in shape to the elongated cells with cytoplasm at both ends of the nucleus described in Table 3.

3.4.2 Long term cultures

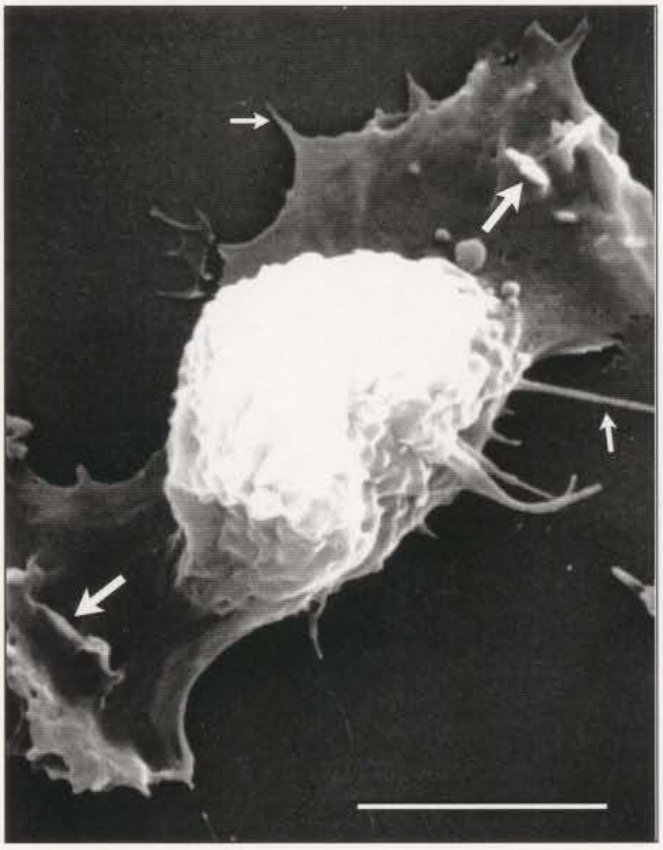
Scanning electron micrographs of cells 8 days in culture are seen in Figure 12. The cells seen in Figure 12A appear very flat, unlike those seen in previous Scanning electron micrographs of single isolated cells (Figure 11). Although the cells in Figure 12 are beginning to grow together to form a confluent layer of cells, numerous spaces can still be seen between cells (arrows - Figure 12A). Those areas where the cells have grown in contact with each other, the cell boundaries cannot be seen clearly, making it very difficult to distinguish individual cells in confluent cultures (small arrows - Figure 12B).

However, not all cells at this stage appear flat. Figure 12B shows a cell from the same culture as those seen in Figure 12A. Although the cell in the middle of the micrograph has begun to contact other cells (small arrows), it has retained the elevated central nuclear

Figure 11: Scanning electron micrographs of winter flounder gill epithelial cells in 24 hr culture. Small arrows show thin, cytoplasmic extensions. Large arrows show vertical protrusions on the cell surface (Figure 10A scale bar = 6.9 μm; Figure 10B scale bar = 4.0 μm).

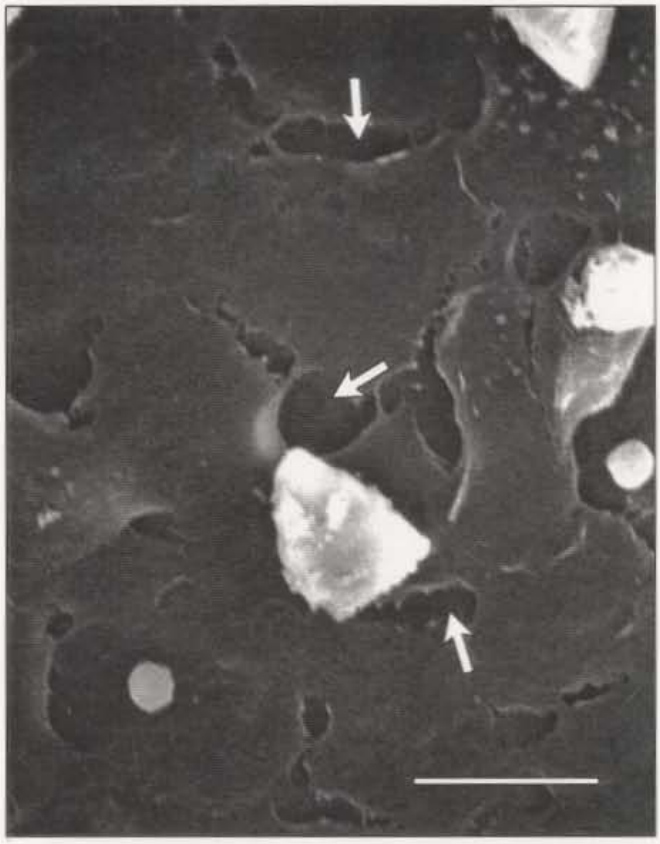


A

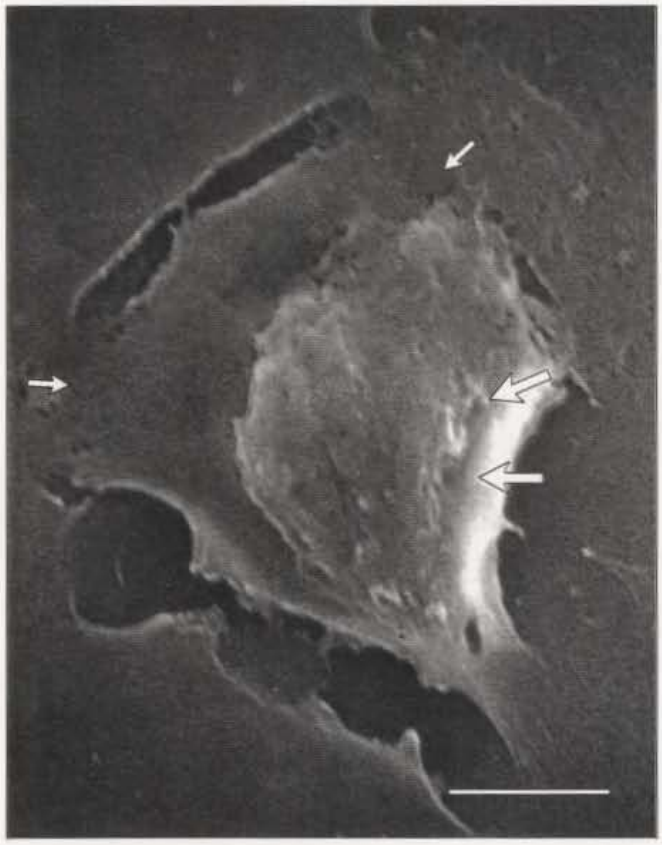


B

Figure 12: Scanning electron micrographs of winter flounder gill epithelial cells eight days in culture. Arrows in 12A show spaces between growing cells. Large arrows in 12B show an elevated nuclear region while small arrows show areas of cell contact (Figure 12A, scale bar = 16.0 μm; Figure 12B, scale bar = 4.0 μm).



A



B

region (large arrow) that is seen in single isolated cells (Figure 11) and at this point, has not flattened out like the cells seen in Figure 12A.

Other scanning electron micrographs were taken of cells that had been in culture 23 days and had grown into a confluent monolayer of cells. These confluent cells were very flat (as seen in Figure 12A) and individual cells could not be distinguished. These scanning electron micrographs were therefore not included because they did not show any additional information.

4. Discussion

4.1 AFP produced by cultured gill cells

The results of the present study demonstrate that cells isolated from the gill epithelium of the winter flounder (*P. americanus*) possess type I AFP. Other investigators have cultured pavement cells from other teleost fish including rainbow trout (Pärt *et al.*, 1993) and sea bass (Avella *et al.*, 1994), but up to this point, no cultures of winter flounder gill epithelial cells have been made. The presence of AFP in these cultured cells provides an opportunity to study the properties and regulatory mechanisms of skin type I AFP in the gill.

Results of AFP detection experiments of winter flounder gill cell cultures suggest that both the skin and liver type I AFP are present in these cells. Both cultures that were tested using antiserum to skin type I AFP were positive. Similarly, cultures that were tested using antisera to liver type I AFP, including WF and Recom antisera, were positive (90 and 100% respectively; Table 3). However, the WF2 antiserum used to test the presence of liver type I AFP did not produce results similar to those of the WF antiserum. These results suggest that although these gill cells tested positive for liver type I AFP, there was a difference in antigen sites recognized by these different antisera (as discussed below). None of the cultures that were tested with the SR2 antiserum were positive for the presence of type II AFP (Table 3). Similar results were seen in cultures where the primary antiserum was omitted (negative control).

The results of the present study are in contrast to other studies that suggest expression of liver type I AFP may be confined to the liver. Using *in situ* hybridization to detect mRNA for skin type I AFP, Gong and colleagues (Gong *et al.*, 1992, 1995) found skin type I AFP

message in many winter flounder tissues, whereas later studies by Gong *et al.* (1996) report that the expression of liver type I AFP mRNA was restricted to the liver, and to a lesser extent, the intestine. In the present study type I AFP was detected in winter flounder gill cells using polyclonal antisera directed against the liver AFP. Polyclonal antisera, like those used here, not only include a relatively high proportion of irrelevant antibodies from the host animal (i.e., rabbit), but are also characterized as containing multiple antibodies directed against different regions (epitopes) of the antigen (i.e., type I AFP) (Harlow & Lane, 1988; Goding, 1996). Thus, antigens that have a high degree of homology between them may induce the production of antibodies that bind to conserved regions of both antigens. Sequence analysis of type I AFP cDNA isolated from the skin of the winter flounder by Gong *et al.* (1996), found 72.1 - 82.2% homology with the liver type I AFP DNA, resulting in skin and liver AFPs that are similar. It is most likely that one or more antibodies in the antiserum made against the liver AFP recognizes an identical or very similar region of the skin AFP, which would subsequently result in the gill cells testing positive for the liver AFP.

The poor response of the cells to the WF2 antiserum indicates there was variation in the type I AFP antisera used. Of the cultures examined using the WF antiserum, 90% tested positive for liver type I AFP whereas only 25% of these cultures tested positive using the WF2 antiserum. Although the antisera were made in the same fashion, they were produced at different times in different animals, thus it is possible that the immune response of the animal producing the WF2 antiserum was not as strong as that of the animal which produced the WF antiserum. It is quite possible that an impurity of the liver AFP elicited a strong immune response in the rabbit, one that may in fact be much more immunogenic than that of

the liver AFP itself (Goding, 1996). This may have led to a diminished or reduced response when using the antiserum to test for the presence of skin type I AFP. It is also possible that these two polyclonal antisera recognized different sites of the skin type I AFP protein. The complications associated with polyclonal antisera may have been avoided if a monoclonal antibody was used. Monoclonal antibodies are highly specific, not only for the immunizing protein, but for a particular epitope within the protein (Goding, 1996). A liver type I monoclonal antibody would not be cross-reactive with the skin type I AFP if it were developed to a specific region of the liver AFP not present in the skin AFP. Otherwise, a monoclonal antibody to liver type I AFP may still elicit a cross-reaction with the skin AFP if the epitope is conserved in both skin and liver AFPs.

The results obtained when treating the cultures of winter flounder gill cells with various antisera indicate that the skin type I AFP is present in these cells, however it is unclear as to exactly where the skin AFP is located. The cells were permeablized with Tween 20 to allow the antisera to reach the skin type I AFP that may be located within the cell, however a similar result would be obtained if the skin AFP were expressed as a surface protein or secreted.

4.2 Regulation of skin type I AFP in winter flounder

The discovery that type I AFP was located in several other tissues of the winter flounder besides the liver (Gong *et al.*, 1992) is important not only for understanding how and where these proteins function, but also how they are regulated.

Gong *et al.* (1995) examined the regulatory mechanisms of AFP expression in both

liver and non-liver tissues. The relatively large seasonal difference in liver AFP mRNA levels was known to be regulated by photoperiod via the hypothalamo-hypophyseal axis (Fletcher *et al.*, 1989), however examination of non-liver tissues showed only a moderate seasonal variation (5 - 10 fold difference) in AFP mRNA levels that was not affected by hypophysectomy (Gong *et al.*, 1995). These observations prompted Gong and colleagues to suggest that liver and non-liver AFP mRNAs were not transcribed from identical sets of genes and that there are two groups of AFP genes in the winter flounder genome. It was not until Gong *et al.* (1996) isolated a family of skin type AFP cDNA genes from the winter flounder genome that the presence of two different AFP genes was confirmed.

Photoperiod and temperature are the only environmental factors that have been shown to have an affect on liver AFP mRNA. It is evident that photoperiod does not play a significant role in the regulation of skin AFP (Gong *et al.*, 1995), however water temperature may contribute to the seasonal fluctuation of skin AFP levels. It is believed that temperature has a post-transcriptional affect on mRNA stability with low temperatures increasing the half-life of liver AFP mRNA (Gong *et al.*, 1995). Low temperatures have been shown to be necessary for the seasonal accumulation of liver AFP mRNA (Vaisius *et al.*, 1989) while higher temperatures lower the amount of AFP mRNA (Lindquist & Petersen, 1990). The mechanism of temperature on the stability on skin AFP mRNA has not been determined. However, because of the similarity of skin and liver AFPs, it is reasonable to assume that temperature would have a similar effect on skin AFP mRNA as it has on liver AFP mRNA, however quantitative testing is needed before any definitive conclusions can be drawn about the possible regulatory effect of temperature on skin AFP levels. The method of gill cell

isolation and culture provided in this thesis could provide a means of testing the effect of temperature on AFP mRNA levels.

Miao and colleagues (Miao *et al.*, 1998) examined the mechanisms controlling the expression of the skin type AFP in winter flounder and found that the skin and liver AFP genes contained two important differences within their only intron which appear to lead to differential regulation, despite their sequence similarity. Firstly, the skin type intron has a 241 - bp fragment not present in the liver type AFP gene. Secondly, the skin type intron has a TA insertion within Element S, a region corresponding to Element B of the liver type AFP intron. Element B is responsible for the enhancer activity of the liver type intron because it binds CCAAT/enhancer binding protein (C/EBPα), while the TA insertion of Element S in the skin type intron essentially destroys the C/EBPα binding specificity. The binding of C/EBPα to Element B is believed to mediate the liver-specificity of the liver type intron, whereas the inability of C/EBPα to bind to Element S is thought to be responsible for the ubiquitous expression of skin type AFP mRNA (Miao *et al.*, 1998). These observations suggest that the skin type AFP gene is constitutively expressed in the winter flounder. Perhaps the post-transcriptional effect of temperature on AFP mRNA may be solely responsible for the moderate seasonal variation of skin type AFP observed in non-liver tissues. However, the possibility that the 241 - bp fragment of the skin type intron might also contain *cis*-acting sequences that interact with transcription factors to regulate skin type AFP expression is currently under investigation (Miao *et al.*, 1998).

4.3 Localization of skin type I AFP in the winter flounder gill

The localization of the skin type I AFP in these gill cells appears as if this is an intracellular AFP, however the current method of testing does not exclude the possibility that the AFP may be expressed as a surface protein or is secreted using an alternate pathway independent of the endoplasmic reticulum (Mignatti *et al.*, 1992).

Examination of non-liver AFP mRNAs from winter flounder by Gong *et al.* (1996) showed the skin type AFP lacked both pre- (secretory signal) and prosequences, unlike the liver AFP that possesses a secretory signal sequence required for the secretion of the protein into the blood, thus suggesting an intracellular role for the skin type AFP. Immuno-histochemistry studies using winter flounder gill by Murray *et al.* (1997) showed that the skin ty3.3pe I AFP was distributed throughout the cytoplasm of individual gill cells leaving the nucleus free of any protein. There was also no significant amount of AFP within the surrounding connective tissue and extracellular areas, providing further evidence that the skin AFP in winter flounder gill has an intracellular function. However, the distribution of skin AFP in the winter flounder epidermis appears somewhat different. Murray *et al.* (1999) demonstrated the AFP of skin epithelial cells was closely associated with the cell membrane and the extracellular matrix. These results suggest the skin AFP found in gill cells and skin epidermal cells may function differently, necessitating further examination of tissues expressing skin type I AFP.

Results of AFP detection in winter flounder gill cells in the current study indicate the skin AFP is located intracellularly based on the distribution of reaction product observed from these cells. Figures 4 - 6 show cells that appear to have the skin AFP distributed throughout the cell. The AFP appears to be located predominately around the periphery of the central

nuclear region, consistent with an intracellular distribution of the protein. If the results had shown a relatively even distribution of reaction product over the entire cell, then it would have suggested the AFP was expressed as a surface protein. Although not tested in the present study, it would be possible to test a surface versus intracellular expression of skin type I AFP by treating the cultured cells with each of the active antisera (WF, Recom & Skin antisera) using the procedure previously outlined without Tween 20. If the antisera is not allowed to penetrate the cell (omission of Tween 20) and the procedure produces results like those seen in Figures 4 - 6, then the AFP would be located on the cells surface. If the results resemble those seen in Figures 5 - 7, then the AFP would almost assuredly be intracellular.

The possibility that the skin type AFP found in these gill cells is also secreted has not been addressed. This however, could be determined by examining the thermal hysteresis activity of the media used to culture the cells. If the culture media exhibits higher antifreeze activity after being incubated with these gill cells than it did before it was added to the cultures, it would be a good indication that these cells are capable of secreting AFP into extracellular spaces.

4.4 Function of skin type I AFP in winter flounder

The discovery of type I AFP mRNA in many winter flounder tissues, and the subsequent discovery that the liver and skin type I AFPs are produced from two genes, has raised questions as to the function of the AFP expressed in non-liver tissues. As previously discussed, the mature skin AFP is synthesized without a secretory signal suggesting it has an intracellular function. Instead of acting as an antifreeze that is circulated by the blood, these

cytosolic skin AFPs might act as a barrier to ice propagation through the skin. Valerio *et al.* (1992) studied the effectiveness of winter flounder skin in blocking ice crystal passage through the skin and found that not only was the skin effective in inhibiting ice propagation, but that the skin's ability to block ice from penetrating the skin was improved with the addition of AFP. Additional information that suggests fish skin is an important site of antifreeze activity comes from studies by Valerio *et al.* (1990) (European shorthorn sculpin - *Myoxocephalus scorpius*) and Schneppenheim & Theede (1982) (cunner - *Tautogolabrus adspersus*) that show AFP expression in the skin, but not in the blood serum. Gong *et al.* (1996) found skin type AFP mRNA present in all winter flounder tissues tested but found the exterior tissues (skin, scales, fins and gills) to express the highest levels of skin type AFP mRNA. These exterior tissues exhibited similar levels of skin type AFP mRNA, whereas the other tissues tested only showed 1 - 10% of the level expressed in these exterior tissues. The relatively high expression levels of the exterior tissues may be necessary for protection from freezing whereas the interior tissues of the winter flounder (liver, stomach, kidney, spleen, heart and intestine) might not need such expression levels since it is likely that they are provided freeze protection from the circulating liver type AFP. During winter months, there is a decrease of blood flow to the skin (Gong *et al.*, 1992). It was also demonstrated by Burton & Fletcher (1983) that the winter flounder epidermis is separated from the vascular tissue by a thick layer of connective tissue thus increasing the difficulty of the liver AFP to diffuse across the dermis from the blood to the skin. The ability of the winter flounder to synthesize relatively high levels of skin AFP in the exterior tissues makes them less reliant on circulating liver AFP and appears to give them a first line of defence against freezing.

If the skin AFP plays a role in freeze protection, how does it function? Although the skin of the winter flounder acts as an effective barrier against ice crystal growth through the skin, it is unlikely that the skin AFP functions as a deterrent to ice formation within the cell. Ice formation within winter flounder epithelial cells is unlikely due to the spatial requirements for ice crystal formation and the nature of the cell membrane itself (Valerio *et al.*, 1992). Thus, ice propagation through the skin is likely confined to the intercellular spaces between epithelial cell layers (Fletcher *et al.*, 1998). An AFP located within these intercellular spaces could account for the effectiveness of the skin of winter flounder, and other fish species, to block the formation of ice through the skin in icy waters. Although the skin AFP was previously thought to function intracellularly, it seems reasonable to think that this AFP plays a role outside the cell in these intercellular spaces. Although this class of proteins has no secretory signal, it is possible that the skin type AFP may be secreted into the intercellular spaces of the skin via a mechanism of exocytosis that is independent of the endoplasmic reticulum-Golgi complex as described by Mignatti *et al.* (1992). Murray *et al.* (1997) observed the distribution of AFP in winter flounder skin. The AFP produced in skin epidermal cells had a close association with the cell membrane and extended into the extracellular matrix outside the cell. Besides providing an obstacle for the passage of ice through the skin, it is likely that these skin AFPs would also function in these intercellular spaces to reduce damage to epithelial cells, such as those of the gill, from ice formation (Fletcher *et al.*, 1998).

Apart from the role of skin AFP in freeze prevention, the ubiquitous nature of the skin AFP gene in the winter flounder suggests that it also has an important role in cold adaptation.

During periods of hypothermia, Hochachka (1986) claims that metabolic and membrane functions become decoupled leading to a rise in intracellular calcium and consequently cell death. However, he proposed that metabolic activity and membrane function can be coupled at lower temperatures by decreasing the permeability of the cell membrane. Thus, these AFPs may operate by having a stabilizing effect on the cell membrane as well as those of the internal organelles by reducing cell membrane permeability. Rubinsky *et al.* (1990) studied the effects of incubating pig oocytes (normally sensitive to hypothermic temperatures) with a mixture of different molecular weight AFGPs at $4^{O}C$. They found that the mixture of AFGPs protected the structural integrity of the oolemma and inhibited ion leakage across the oolemma at hypothermic temperatures. Similar studies by Rubinsky *et al.* (1992) and Negulescu *et al.* (1992) looked at AFPs effect on ion channels. Rubinsky *et al.* (1992) showed that both Ca^{2+} and K^{+} currents of pig granulosa cells were suppressed in the presence of type I AFP while Negulescu *et al.* (1992) demonstrated that type III AFP was effective in preventing the passive influx of Ca^{2+} into rabbit parietal cells. While these studies demonstrate the stabilizing effects of AFPs on ion channel behavior, they do not indicate what effect, if any, these AFPs have on the membrane itself. Cells may become injured while being cooled by undergoing thermotrophic phase transitions. During this transition between the liquid crystalline to gel phase, the membrane becomes leaky resulting in the loss of intracellular contents (Quinn, 1985). Hays *et al.* (1996) demonstrated that as artificially created bilayers containing a trapped marker were cooled and warmed through their transition temperature, they leaked up to 50 % of their contents. However the addition of AFGP prevented the leakage of the trapped marker by up to 100% (Hays *et al.*, 1996). A similar study by Talbin *et al.* (1996)

that examined the membrane phase transition and cold-induced activation of human platelets showed that the addition of as little as 1.0 mg/ml of AFGP was able to decrease platelet activation at 4°C. It is doubtful that the AFP located in the gill cells of the winter flounder functions by preventing leakage from phase transition, however the above studies further demonstrate the stabilizing effect AFPs can have on membranes.

The question still remains as to where the skin AFP provides this protective effect. Although the studies referenced above discuss the effect of several different AFPs and AFGPs on the exterior surface of membranes, as would occur with a secreted AFP, the possibility that the skin AFP interacts with the interior surface of the cell membrane is very likely since most evidence thus far indicates that the skin type AFP found in the winter flounder gill is located intracellularly. Thus, it would be interesting to examine the effect of skin type I AFP on membrane function.

4.5 Identification of gill cells in culture

This is the first description of gill epithelium cells from winter flounder to be grown in culture. Investigators Pärt *et al.* (1993) and Avella *et al.* (1994) have isolated gill epithelial cells from both fresh water (rainbow trout - *Oncorhynchus mykiss*) and marine fish (sea bass - *Dicentrarchus labrax*) species respectively, using slightly different procedures but with similar results. Pärt *et al.* (1993) found that their original trypsin digest contained a mixture of different cell types, including red blood cells. However after changing the culture medium 24 hrs after isolation, any unattached cells washed away. Using the fluorescent dye DASPMI to identify any mitochondria-rich chloride cells in cultures 24 hrs after seeding and at

confluence, they concluded that mitochondria-rich chloride cells did not attach under these culture conditions. Similar results were obtained by Avella *et al.* (1994) when checking for the presence of chloride cells in their monolayer cell cultures using the flourescent probe DASPEI. They deduced that if chloride cells were present in the explant during the culture, none of them grew or proliferated in the cell monolayer. It is believed that chloride cells are not capable of surviving the culture conditions possibly because the culture medium lacks essential elements for survival, such as cortisol as indicated by McCormick (1990). Based on these results, they concluded that the cells that remained in the dish were a homogeneous population of pavement cells. The isolation of a homogenous population of pavement cells by these investigators is not surprising since the pavement cell is the most predominant cell population of teleost brachial epithelium regardless of the salinity of the surrounding environment (Avella *et al.*, 1994; Avella & Ehrenfeld, 1997).

Perhaps the best method these investigators used to identify the gill cells in culture is the use of scanning electron microscopy (SEM). Both studies showed the cells that grew and formed a contiguous monolayer in culture contained a complex system of microridges. These microridges are characteristic of pavement (respiratory) cells found in teleosts and are typically not observed on any other gill epithelial cell. This, along with transmission electron microscopic (TEM) observations conducted by Avella *et al.* (1994), indicate that the contiguous monolayers of cells produced by both culture techniques are comprised of pavement (respiratory) gill cells.

In an attempt to identify the cells isolated and cultured using the procedure outlined in this thesis, the cultures were morphologically examined using light, scanning electron and

fluorescence microscopy.

4.5.1 Light Microscopy

The cells seen in Figure 2 can be grouped into three predominant cell shapes (round, crescent and elongated). These cells that have plated onto coverslips are believed to be of the same type despite their obvious differences in shape and size because all shapes test positive for the presence of type I AFP. Some cultures however, contained smooth, oval red blood cells (rbc arrow - Figure 2B) that are clearly different from the majority of the other cells present. Pärt *et al.* (1993), Avella *et al.* (1994) and Pärt and Bergström (1995) all describe such red blood cells as being present in their respective gill epithelium isolates, but they do not attach to the culture dishes and are later removed through washing or changing the culture media.

After cell isolation, Pärt *et al.* (1993) described single cells in 24 hrs culture as being elongated and are similar to those cells observed in Figure 2. Pärt *et al.* (1993) are the only investigators to describe single, isolated gill cells, however the isolated cells are examined using a low magnification and only a brief description of single cells is given.

The descriptions of confluent gill cells by the above investigators are again similar to contiguous cells shown in Figure 3. Gill cells isolated by Avella *et al.* (1994) are reported as being flat, epithelioid shape. They later describe the cells as being flat, thin and usually polygonal when observed using SEM. Pärt *et al.* (1993) report gill cells in small scattered colonies had a rounded appearance, a departure from their previous elongated shape. In the present study, gill cells of the winter flounder were found to form similar aggregations of cells. Single cells that were once crescent and elongated shape had a more rounded

appearance when in aggregates. Those cells described as being round appeared to have become larger or perhaps flatter (Figure 3).

As previously stated, few dishes contained colonies of contiguous winter flounder gill cells, and those cultures that did contain aggregate cells required more time than cultures described by Pärt *et al.* (1993) which after 3 days in culture, covered most of the culture dishes in colonies of 5-50 cells. Under the current culture conditions, the gill cells of the winter flounder required up to as much as 23 days to form contiguous colonies of cells. One reason for this difference could be the numbers of cells required for the formation of confluent layers. No colonies of cells were seen in dishes that had less than ~ 35 cells per 62500 μm^2. It is also possible that the culture conditions, although appropriate for the maintenance of gill cells, were not suitable for the formation of a confluent monolayer of cells.

Despite the differences in length of time needed to obtain confluent cells, the similarity between these cultured winter flounder gill epithelial cells and those described by Pärt *et al.* (1993) and Avella *et al.* (1994) indicates that the cells cultured here are pavement cells. Further evidence that suggests these cultured cells are pavement cell comes from immuno-histochemical studies by Murray *et al.* (1997). AFP producing cells in the gill showed a distribution pattern similar to that of pavement cells while experiments using Na^+/K^+ ATPase to identify chloride cells showed a different distribution pattern (Murray *et al.*, 1997). In an attempt to provide further evidence for the identification of these cells as pavement cells, SEM was performed on cells 1, 8 and 23 days in culture.

4.5.2 Scanning electron microscopy

It was thought that scanning electron micrographs of these single gill cells would show

a system of microridges which would provide strong evidence that these winter flounder gill cells were in fact pavement cells. Winter flounder gill cells that have been isolated and maintained in fish saline for 24 hrs are shown in Figure 11. According to the cell shapes outlined in Table 3, these cells would be considered elongated shape, with a rough, elevated central nuclear region and flat outer regions. However, there was no evidence of a system of microridges in these cells in short term culture.

To determine if the absence of microridges after 24 hrs in culture was due to some aspect of the isolation procedure, SEM was used to observe cells 8 days (Figure 12) and 23 days (data not shown) in culture. Again, there was no evidence of microridges present as can be seen in 8 day old cells in Figure 12. Perhaps if more cultures had been examined using SEM, some surface organization resembling microridges or microvilli may have been observed.

Avella *et al.* (1997) demonstrated that the differentiation of the microridges of sea bass pavement cells corresponded with the cell culture conditions, namely the nature of the supplemented serum. They showed cells cultured with fetal bovine serum (FBS) had irregular microridges or reduced ridges that appeared to be microvilli. Other cells cultured with FBS lacked any apical surface arrangement and had an almost smooth surface. In the present study, the very flat appearance of contiguous gill cells may be a result of using FBS in the culture medium instead of a serum such as that prepared from the winter flounder. An attempt was made to produce winter flounder serum for the culture of gill cells, however the serum proved very difficult to filter through 0.22 μm Millipore filter so FBS was used in the culture medium. The cells cultured for 24 hrs seen in Figures 11A & B show short vertical

protrusions on the cells surface that are not seen in long term cultures. It is possible that these protrusions are remnants of microridges present in these cells prior to isolation which where subsequently lost because a suitable serum was not present in the culture medium for the maintenance of microridges like those typically seen in pavement cells.

4.5.3 Fluorescence microscopy

Chloride cells found in teleost gill epithelia contain numerous mitochondria. One of the most common methods to identify chloride cells is the use of a fluorescent probe such as DASPEI that allows mitochondria-rich cells to be visualized using fluorescence microscopy (Marshall & Nishioka, 1980). DASPEI was used in the present study to determine if these winter flounder gill cells grown in culture contained numerous mitochondria like that typical of the chloride cell. As seen in Figure 10, cultured winter flounder gill cells that have been treated with DASPEI fluoresced against the dark background suggesting that these isolated gill cells are mitochondria-rich like that of the chloride cell. Previous studies that isolated and cultured gill epithelial cells from teleost species showed that only pavement cells were able to attach and proliferate in the culture conditions provided as determined using mitochondrial fluorescent probes (DASPEI, DASPMI) and SEM (Pärt *et al.*, 1993; Avella *et al.*, 1994; and Pärt & Bergström, 1995; Avella & Ehrenfeld, 1997). Furthermore, as previously cited, cortisol is believed to be necessary for the maintenance of chloride cells in culture (McCormick, 1990). These facts suggest that it is unlikely that the winter flounder gill cells cultured here are chloride cells, however results from DASPEI staining suggest that these are mitochondria-rich cells, a characteristic thought only to be exhibited by chloride cells in brachial epithelium. Because there is no literature available concerning the distribution of

mitochondria-rich cells of the winter flounder gill epithelium, it is possible that not only are gill chloride cells plentiful in mitochondria, but the pavement cell of the winter flounder gill epithelium is as well. Although pavement cells of all teleost species previously studied were shown not to be rich in mitochondria (Pärt *et al.*, 1993; Avella *et al.*, 1994; and Pärt & Bergström, 1995; Avella & Ehrenfeld, 1997), the pavement cell of the winter flounder gill may require numerous mitochondria to produce the significant amounts of skin AFP as detected in gill cells observed in the present study and by Murray *et al.* (1997), regardless if this AFP is located internally or secreted into the intracellular milieu.

Chloride cells of the teleost gill also have a high content of Na^+/K^+ ATPase (Pärt *et al.*, 1993). Murray *et al.* (1997) used a monoclonal antibody for Na^+/K^+ ATPase to view chloride cells in winter flounder gill tissue sections and observed a distribution of Na^+/K^+ ATPase positive cells consistent with chloride cell distribution in the gill, but different from the distribution of skin AFP positive cells. Perhaps the best way to determine which cells of the winter flounder gill are AFP producing, mitochondria-rich, and chloride cells is to conduct parallel experiments using skin AFP antisera, DASPEI and monoclonal Na^+/K^+ ATPase antibody on gill tissue sections to determine the distribution of cells that react to each of these treatments. One would expect to see a widespread distribution of DASPEI positive cells in the winter flounder gill if the pavement cells are mitochondria-rich, since both pavement and chloride cells would presumably test positive.

4.5.4 Cell Culture

In an effort to isolate, maintain and grow gill epithelial cells from a marine species, a

method was developed based on a procedure used by Pärt *et al.* (1993). This modified procedure had to account for the difference in osmolarity between saltwater and freshwater fish. This was accomplished by substituting fish saline, a solution adjusted to the osmolarity of sea water, for PBS. The explant technique used by Avella *et al.* (1994) was attempted, however this method did not appear to work as well as the adapted Pärt *et al.* (1993) procedure. The technique employed by Pärt *et al.* (1993) relied on the use of the enzyme trypsin to release the cells from the gill, which lead to a large variety of cell types in the culture dish and large amount of mucus. In order to view individual cells that have adhered themselves to the coverslip of the culture dish, considerable washing with fish saline was needed and this washing presumably removed many plated gill cells. Another possible problem that may be attributed to the use of trypsin to isolate these adherent cells is its possible affect on cell morphology. Avella *et al.* (1994) however, does not use trypsin to release the cells of interest from the gill. Instead, they use a method where the gill filaments are teased into small tissue fragments and a confluent monolayer of pavement cells is formed from both cell migration and cell division. Subsequent attempts to culture winter flounder gill cells may use the procedure outlined here in this thesis, however an attempt should be made to use the explant technique with some possible modifications to take advantage of its simplicity.

4.6 Summary

Although several investigators have successfully isolated and cultured branchial epithelial cells from such teleost species as rainbow trout and sea bass, the current study is

the first description of cells that have been isolated and cultured from the gill epithelium of winter flounder. Although these cells did not display the characteristic microridges typically seen in pavement cells, they attached to coverslips and were capable of growing into confluent monolayers, similar to gill epithelium pavement cells cultured by previous investigators. It is possible that the surface morphology of these cells (i.e. microridges) may have been affected by either the cell isolation procedure, the culture media or both. These cells were also shown to be mitochondria-rich, typical of chloride cells, however the current culture conditions are not thought to be conducive for chloride cell growth.

Using both liver and skin AFP polyclonal antisera, it was demonstrated that these cells produced an AFP that was reactive with both antisera. Due to the close similarity between skin and liver AFPs, it is thought that the liver AFP antisera cross-reacted with the skin type I AFP present within these cells. The distribution pattern of AFP exhibited by these cells suggest that the AFP was located intracellularly and not expressed as a surface protein, however the present study did not determine if these cells were capable of AFP secretion.

The role skin AFP plays in the gill has not yet been determined. The ability to culture and maintain AFP producing gill cells from the winter flounder may prove useful in future studies attempting to determine how these skin AFPs function and how they are regulated. These answers will not only advance our understanding of the skin type I AFP of winter flounder, but could also provide insight on how these and other AFPs may be used for medical or commercial purposes.

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