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PATHOBIOLOGY OF MYCOBACTERIA IN STRIPED BASS (*MORONE SAXATILIS*)

A Dissertation
Presented to

The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirements for the Degree of
Doctor of Philosophy

By
David T. Gauthier
2004
This dissertation is submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

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DEDICATION

This work is dedicated to my parents, who have supported me through everything.
ACKNOWLEDGMENTS

Through the course of this work, I have received the guidance and assistance of many people both within and without the VIMS community. Foremost, I would like to thank my major advisors, Drs. Wolfgang Vogelbein and Stephen Kaattari, as well as the other members of my advisory committee, Drs. Howard Kator, Christopher Ottinger, and Peter VanVeld. I would also like to thank Dr. Emmett Shotts, who served on my advisory committee until his retirement in 2002. In addition to my committee members, thanks to Drs. Kim Reece and Jeff Shields for allowing me to work in their laboratories.

I am deeply grateful for the expert technical assistance I have received in various laboratories at VIMS. Initial isolation and characterization of the *Mycobacterium* spp. used in these studies was performed by Martha Rhodes, who also provided the splenic bacterial density data in Chapter 1. Martha has taught me much of what I know about working with mycobacteria, a group of organisms which seems to delight in being difficult to do anything with. I would like to thank Patrice Mason for teaching me the fine points of electron microscopy. I can only hope that one day my preparations will look as good as hers. I owe special thanks to Pat Blake, who cheerfully processed huge amounts of histological material for my various projects. I would also like to thank Dana Booth, Luke Iwanowicz, Ilsa Kaattari, Julie Stubbs, and Dave Zwemer for assistance, instruction, and generally putting up with me.

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I have benefitted greatly from collaboration with fellow students during my time at VIMS. Erin Burge has been both a good friend and colleague, and I owe him much for making this degree more productive and enjoyable. I also thank Chris Earnhart for our collaboration on *Perkinsus* immunogold labeling, as well as co-authorship on the resulting manuscript.

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Finally, I would like to thank all the friends who have kept me sane, especially Erica Westcott, whose love and friendship has carried me through.
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ABSTRACT

Striped bass (*Morone saxatilis*) in Chesapeake Bay, USA, are experiencing an epizootic of mycobacteriosis. This disease, caused by bacteria in the genus *Mycobacterium*, causes granulomatous lesions of the skin and viscera. Diseased fish are often emaciated, and fish with skin lesions may be significantly disfigured. The overall goal of this work was to examine aspects of the pathobiology of mycobacteria in striped bass via laboratory exposure studies and *in vitro* cellular assays.

Striped bass were injected intraperitoneally with a sublethal dose of *Mycobacterium marinum*, *M. shottsii*, or *M. gordonae* and sampled for histology and bacteriology to 45 weeks post-injection (p.i.). Fish injected with *M. marinum* developed granulomas in the mesenteries, spleen and anterior kidney. Acid-fast bacilli (AFB) were rare in the initial phase of disease, whereas granulomas at 8 weeks p.i. and later frequently contained large numbers of AFB. Secondary disease was observed in some fish between 26 and 45 weeks p.i., with granuloma disintegration, severe inflammation, and elevated splenic bacterial densities. Fish injected with *M. shottsii* or *M. gordonae* did not develop severe pathology. Relatively small granulomas were formed in the mesenteries, but were not observed in the spleen or, with one exception, anterior kidney. *M. shottsii* and *M. gordonae* both established persistent splenic infections.

The ultrastructure of developing *M. marinum* granulomas in experimentally infected striped bass was examined. Formation of large macrophage aggregations containing intracellular bacilli was observed within the peritoneal cavity shortly after injection. *M. marinum* were always contained within phagosomes, and apparent phagolysosomal fusion was frequently observed. Epithelioid transformation of macrophages with desmosome formation was observed. Ultrastructural observation of bacilli within granulomas agreed with histologic findings, indicating that large numbers of *M. marinum* at later time points derived from smaller numbers of bacilli surviving within granulomas, rather than from chromophobic or alternate bacillary forms.

Interaction between macrophages and intracellular *M. marinum in vitro* was examined ultrastructurally and with a quantitative bactericidal assay. Phagosomes containing *M. marinum* were fused at high rates by pre-labeled lysosomes. No differences in lysosomal fusion rates were observed between phagosomes containing live or heat-killed *M. marinum*. Despite presence of lysosomal contents within phagosomes, intracellular *M. marinum* remained viable for the duration of the assay (72 hours). Heat-killed *M. marinum* were similarly resistant to lysis within phagolysosomes.

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PATHO BIOLOGY OF MYCOBACTERIA IN STRIPED BASS
(MORONE SAXATILIS)
Mycobacteriosis of fishes: historical background

Before the turn of the 20th century, a tuberculous disease associated with acid-fast bacilli was described in carp (*Cyprinus carpio*) kept in a pond receiving effluent from a tuberculosis ward (Bataillon et al., 1897). This discovery led to several studies of whether mycobacteria infecting mammals could be adapted to fishes, and vice versa (reviewed by Parisot, 1958). *Mycobacterium marinum*, currently the most commonly described species of mycobacteria affecting fishes, was first isolated from several species of captive tropical marine fishes in 1926 (Aronson, 1926). *Mycobacterium fortuitum*, another important species of mycobacteria affecting fishes, was first isolated from the neon tetra (*Paracheirodon innesi*) in 1953 (Ross & Brancato, 1959).

Since these early studies, mycobacteriosis has been recognized as one of the most common diseases affecting stocks of cultured and wild fishes worldwide. A review in 1963 described 150 species of fishes, representing 40 families, affected by mycobacteriosis (Nigrelli & Vogel, 1963). Since this report, a number of susceptible species have been added to the list, including representatives from tropical freshwater (Noga et al., 1990; Lansdell et al., 1993), temperate freshwater (Lund & Abernethy, 1978; Daoust et al., 1989), subarctic freshwater (Bruno et al., 1998), tropical marine (Giavenni et al., 1980; Diamant et al., 2000), temperate marine (Arakawa & Fryer, 1984), and
subarctic marine habitats (MacKenzie, 1988). This list of references is by no means exhaustive, and mycobacterial infections in additional species of marine and freshwater fishes are frequently reported. Mycobacteria are ubiquitous in soils and water worldwide, so this cosmopolitan distribution of hosts is not unexpected. Mycobacteriosis has also been reported in several species of invertebrates, including freshwater prawn (Macrobrachium rosenbergii) (Brock et al., 1986), Pacific White Shrimp (Penaeus vannamei) (Mohney et al., 1998), and other penaeid shrimp (Lightner, 1996).

**Mycobacteria**

Mycobacteria, comprising the genus *Mycobacterium*, are pleomorphic, gram-positive, aerobic, acid-fast staining, nonmotile rods, 0.2-0.6 μm in diameter and 1 to 4 μm long. According to the Centers for Disease Control (Atlanta, GA), 82 species of mycobacteria were recognized (http://www.cdc.gov/ncidod/dastb/TB/TB_NTM.htm) as of the year 2000, united by the above characteristics and a unique cell wall that includes high molecular weight (60-90 carbon) long-chain 3-hydroxy fatty acids known as mycolic acids (Draper, 1971; Goodfellow & Magee, 1997). Although structurally similar, mycobacteria differ greatly in their ecological niches, ranging from *M. tuberculosis*, one of the leading causes of human mortality worldwide, to saprophytic soil residents such as *M. terrae* (Wayne, 1966). Mycobacteria are commonly separated on the basis of their rate of growth, with fast-growth species requiring less than 7 days to produce visible colonies on solid agar. Slow-growth species may require several weeks to months to achieve comparable growth. These groups may be further subdivided into
photochromogenic (forming pigment in the light) and scotochromogenic (forming pigment in the dark) strains. Definitive characterization of mycobacterial strains is dependent on biochemical characteristics and DNA sequence analysis. Most studies published to date implicate *M. marinum*, *M. fortuitum*, or *M. chelonae* as agents of piscine mycobacteriosis. Recent work, however, indicates that fishes may be infected with many other species of mycobacteria, including species identical to or resembling *M. neoaurum*, *M. simiae*, *M. poriferae*, *M. scrofulaceum*, and *M. triplex* (Lansdell et al., 1993; Tortoli et al., 1996; Herbst et al., 2001).

### Clinical signs and gross pathology

Piscine mycobacteriosis is predominately a chronic disease that may or may not produce gross clinical signs. All tissues of the fish may be involved, including eyes, gills, visceral organs, musculature, and fins. External clinical signs of mycobacteriosis, when present, include scale loss and dermal ulceration (Snieszko, 1978; Wolke & Stroud, 1978), pigmentation changes (Ross, 1970; Snieszko, 1978), abnormal behavior, spinal defects (Nigrelli & Vogel, 1963), emaciation, and ascites (Bruno et al., 1998). Gross signs of internal infection include enlargement of spleen, kidney and liver, with grey or white nodules scattered throughout the internal organs (Chinabut, 1999). In advanced cases, visceral organs may become fused to the mesenteries. Mycobacteriosis is not typically associated with large-scale mortality in natural settings, although significant losses due to the disease have been incurred in aquaculture (Hedrick et al., 1987; Bruno et al., 1998).
Histopathology

The classic histopathologic manifestation of piscine mycobacteriosis is granulomatous inflammation. Granulomatous inflammation is defined as “a distinctive pattern of inflammatory reaction in which the predominant cell type is an activated macrophage with a modified epithelial-like (epithelioid) appearance.” (Cotran et al., 1999). Epithelioid cells are polygonal, eosinophilic cells with open-faced nuclei, prominent nucleoli, and faintly granular cytoplasm. In mammals, epithelioid cells are derived from mononuclear phagocytes, and differ from true epithelial cells in that they do not possess desmosomes, instead achieving tight cellular packing with interdigitating cytoplasmic projections (Adams, 1974; 1975; 1976). Desmosomes have been found between epithelioid cells of fish granulomas, suggesting that some granulomas in fishes may be formed by different cell types than in mammals (Noga et al., 1989). Mycobacterial granulomas in fishes may be found in all organs and tissues, but are most commonly observed in visceral organs, especially the spleen. A wide range of size and structural organization is seen, ranging from poorly organized inflammation with minimal epithelioid cell formation to the more commonly observed chronic granuloma. Chronic granulomas are composed of concentric layers of epithelioid cells forming a discrete spherical lesion. Caseous necrosis or calcification of the core region may be observed, in a manner similar to that seen in human tuberculosis. A capsule of fibrous material and/or inflammatory cells frequently surrounds fish granulomas.

The classic histological technique for observing mycobacteria in tissue sections is the Ziehl-Neelsen acid-fast stain. This stain relies on the property of the mycobacterial
cell wall to resist acid-alcohol decolorization after staining with carbol-fuchsins, a property not shared by other gram-positive or gram-negative bacteria, with the exception of *Nocardia* spp. Acid-fast bacilli are frequently visible within piscine mycobacterial granulomas, although granulomas containing no detectable acid-fast organisms have been described in wild yellow perch (*Perca flavescens*) (Daoust et al., 1989) and in seabass (*Dicentrarchus labrax*) experimentally infected with *M. marinum* (Colorni et al., 1998). The use of Fite’s acid-fast stain was reported to improve detection of acid-fast bacilli in the former study.

The presence of multinucleate giant cells, characteristic of mycobacterial granulomas in mammals (Adams, 1976) has been variably reported in fishes and has been suggested to be indicative of early lesions (Gomez, 1998). The majority of mycobacterial lesions described in fishes lack these cells (Majeed et al., 1981; Bruno et al., 1998; Colorni et al., 1998) but their presence may be dependent on the species-specific reaction of the host. The inconsistent presence of giant cells has added fuel to the debate of whether mycobacterial infection of fishes should be termed "fish tuberculosis," but recent convention appears to have settled on the more general term mycobacteriosis for this type of infection in fishes (Bruno et al., 1998; Colorni et al., 1998; Talaat et al., 1998).

**Controlled studies of mycobacterial infection in fishes and other poikilotherms**

Much of the literature on mycobacterial infection involves study of spontaneously arising disease in wild or cultured fishes. Relatively few controlled infection studies have
been conducted to date. In an experimental study of intramuscular *M. piscium* infection of plaice (*Pleuronectes platessa*), granulomas were produced in the muscle and the presence of multinucleate giant cells was documented (Timur et al., 1977). Acute mortality with external vent and lateral surface lesions of rainbow trout (*Oncorhynchus mykiss*) was observed after intraperitoneal injection of *M. chelonae* (Arakawa & Fryer, 1984). High mortality from intramuscular *Mycobacterium* spp. injection has been demonstrated in pejerrey (*Odonthestes bonariensis*), with granulomas produced at the injection site and in internal organs within 14 to 30 days of infection (Hatai et al., 1993). Intraperitoneal injection of *M. marinum* in seabass (*Dicentrarchus labrax*) produced granulomas peaking in intensity at 6-8 weeks, followed by varying degrees of disease resolution (Colorni et al., 1998). Chronic granulomatous disease was produced in Atlantic salmon (*Salmo salar*) after infection with *M. chelonae* (Bruno et al., 1998). The first comprehensive dose-response study of piscine mycobacterial infection was conducted using goldfish (*Carassius auratus*) and *M. marinum* (Talaat et al., 1998). In this study, varying dosages of mycobacteria were found to induce either chronic or acute infections, with significant mortalities occurring at dosages of $>10^8$ colony-forming units (CFU) fish$^1$. In a later study, goldfish were infected with *M. fortuitum* and two strains of *M. smegmatis* (Talaat et al., 1999). One strain of the latter proved to be the most pathogenic of the three, producing a systemic granulomatous inflammation typical of other mycobacteria. This result was surprising because *M. smegmatis* is generally

$^1$ *M. piscium* is an obsolete species name. Isolates given this name are likely *M. marinum* (van Duijn, 1981)
considered to be an environmental saprophyte and harmless commensal of mammals, whereas *M. fortuitum* is a known pathogen, especially of immunocompromised animals. These findings suggested that ubiquitous, presumably non-pathogenic mycobacteria may play a role in disease of fishes. Rapidly lethal mycobacteriosis was produced in striped bass (*Morone saxatilis*) after high intramuscular doses of *M. marinum*, and increased disease severity in striped bass relative to tilapia (*Oreochromis* spp.) was demonstrated (Wolf & Smith, 1999). Finally, granulomatous disease histologically similar to that seen in fish and mammals has been produced with *M. marinum* in the leopard frog (*Rana pipiens*) (Ramakrishnan et al., 1997).

**Immune response to mycobacteria in fishes**

Relative to mammals, little is known about the immune response to mycobacteria in fishes. Increased nonspecific immune function (i.e.: superoxide (O$_2^-$) production, lysozyme, and phagocytosis) has been demonstrated in rainbow trout injected intraperitoneally with extracellular products (ECP) of *M. marinum* and *Mycobacterium* spp. (Chen et al., 1996) This study also documented the production of antibodies specific to various proteins of the ECP. A subsequent study obtained similar results in tilapia (*Oreochromis nilotica*) (Chen et al., 1998). A further study explored the antigenicity of ECP components in rabbits, fish, and mice, and found certain proteins to be strongly immunogenic across species (Chen et al., 1997).

An ultrastructural study of *Mycobacterium* spp. interaction with rainbow trout phagocytes was the first to explore the cellular immune response to mycobacteria in fish.
Phagocytosis was found to proceed readily, and intracellular bacteria persisted within phagosomes for the 6 hour duration of the study. The authors of this study stated that fusion of ferritin-labeled lysosomes with phagosomes containing live *Mycobacterium* spp. was inhibited relative to phagosomes containing formalin-killed mycobacteria. The methods for phagolysosomal fusion quantification were not given in that publication, however, and the presence of ferritin within lysosomes was not definitively demonstrated by the included electron micrographs. A more recent study compared the intracellular behavior of *M. marinum* and the nonpathogenic *M. smegmatis* in a monocytic cell line from carp (El-Etr et al., 2001). *M. marinum* entered phagocytes at a much higher rate than *M. smegmatis*, and was more resistant to intracellular killing. Additionally, phagosomes containing *M. marinum* fused with thorium dioxide-labeled lysosomes at lower rates than those containing *M. smegmatis*.

**Transmission**

Transmission of fish-pathogenic mycobacteria is poorly understood. Water is the natural habitat for many species of mycobacteria, so waterborne transmission seems likely, in addition to direct contact with infected fishes. Mycobacteria are known to infect a number of aquatic organisms other than fishes (Nigrelli & Vogel, 1963; Mohney et al., 1998), so potential host vectors are present throughout the food web. Fishes are widely believed to be infected by ingestion of contaminated food products and water (Parisot, 1958; Wood & Ordal, 1958; Ross & Brancato, 1959; Ross, 1963), although this opinion is based largely on empirical observation. Vertical transmission of mycobacteria between
fishes has been suggested (Ashburner 1977), but has not yet been definitively confirmed.

**Zoonotic considerations**

In addition to their known infectivity to fishes, aquatic mycobacteria pose significant zoonotic concerns. *M. marinum* is a well-known pathogen of humans, producing lesions in the skin and deep tissues, especially of the extremities (Collins et al., 1985; Travis et al., 1985; Collins et al., 1988; Lawler, 1994). First described in 1951 (Norden & Linell, 1951), this type of lesion has been repeatedly described in fishermen and aquarists (Zeligman, 1972; Hay et al., 1975) and has been given the common name of fish tank- or swimming pool-granuloma, or “fish-fanciers finger.” More recently, *M. peregrinum* and *M. scrofulaceum* infections have been implicated in the production of similar persistent cutaneous granulomas in aquarists (Ishii et al., 1997). *M. fortuitum* has been found to produce subcutaneous granulomas at insulin injection sites (Pagnoux et al., 1998) and is well known as a pathogen of immunocompromised persons. The importance of fishes as potential vectors of these and other *Mycobacterium* spp. underscores the need to understand the disease process in fishes so that appropriate diagnostic techniques and epidemiologic studies may be developed.

**Life history and economic importance of striped bass (Morone saxatilis)**

The striped bass, or rockfish (*Morone saxatilis*) (Figure 1) is an anadromous teleost fish found along the Atlantic coast of North America, ranging from the St. Lawrence River, Canada, to the St. Johns River, Florida. Introduced populations are also
Figure 1. Striped bass (*Morone saxatilis*) collected from York River, Virginia; male, 581g, 331 mm.
present in the Gulf of Mexico, along the Pacific coast of North America, and in large inland reservoirs. Four main stocks of striped bass are present in U.S. waters: the Atlantic coastal migratory stock, the North Carolina stock, the South Atlantic stock, and the West Coast (Sacramento-San Joaquin) stock (Austin, 1980). The majority of the Atlantic coastal migratory stock uses Chesapeake Bay, USA as spawning and nursery grounds, with spawning taking place in tidal freshwater of rivers when water temperature reaches 8°C in spring (Austin, 1980). Male striped bass tend to mature at approximately 2+ years of age, while females mature later, at age 4+. Females typically begin migrations to sea at age 2-3, while males begin migrations at a later age (Kohlenstein, 1981). Striped bass are sexually dimorphic with respect to size, with nearly all large fish being female. Striped bass are found in a wide range of water temperatures, with a maximum preferred temperature of approximately 23°C (Coutant, 1985). In summer months in Chesapeake Bay when surface water temperatures are well in excess of 23°C, striped bass are typically found in the deeper channels of the Bay.

The striped bass is one of the most commercially and recreationally important fish in Chesapeake Bay. Pound nets, gill nets, haul seines, purse seines, fyke nets, and other methods are used to harvest striped bass commercially, and the species is a traditional favorite of recreational anglers. In the 1970s and 1980s, striped bass stocks underwent sharp declines, prompting enactment of fishing regulations by the Atlantic States Marine Fisheries Commission (ASMFC) and state governments to restrict commercial and recreational striped bass landings (ASMFC, 1981). A limited fishery was re-opened in 1990, and the population is considered recovered, with abundant commercial and

**Mycobacteriosis in striped bass (Morone saxatilis) of Chesapeake Bay**

Recent epizootics of disease have been reported in wild striped bass from Chesapeake Bay. Mortality in fish from Maryland waters of the bay in 1988 was associated with *Streptococcus* spp. infections (Baya et al., 1990). In the summer and fall of 1994, skin and internal lesions appearing on Maryland striped bass were attributed to *Edwardsiella* spp. (Baya et al., 1997). Skin lesions appearing in striped bass throughout the Bay observed beginning in 1997 (Figure 2a) were attributed to a variety of etiologic agents, including the dinoflagellate *Pfiesteria piscicida*. Histological examination of diseased fish, however, revealed acid-fast bacteria associated with granulomatous inflammation of the skin and visceral organs (Figure 2b), observations consistent with infection by *Mycobacterium* spp. (Vogelbein et al., 1999). An epizootiological survey of striped bass in Virginia tributaries of the Chesapeake Bay conducted between 1998-1999 indicated high rates of both dermal and visceral mycobacteriosis in striped bass, with prevalences reaching as high as 28.8 and 62.7%, respectively (Cardinal, 2001). Subsequent surveys have also indicated high rates of dermal and visceral mycobacteriosis in Chesapeake Bay striped bass (Overton et al., 2003; Gauthier unpublished data). These
Figure 2. a) Severe ulcerative dermatitis in wild striped bass collected in Chesapeake Bay near Solomon's Island, MD. b) Granulomatous spleen in wild striped bass collected from York River, VA.
recent surveys have suggested that severe mycobacterial disease is associated with decreased condition factor and emaciation.

Whether mycobacteriosis directly leads to decreased condition factor, or if nutritionally challenged fish are more susceptible to disease, remains a point of contention. Coincident with the strong rebound in striped bass stocks has been a decline in several important forage species (Durell & Weedon, 2003), including Atlantic menhaden (*Brevoortia tyrannus*). This has led to speculation that striped bass populations may not be sustainable at current levels due to lack of food, and that mycobacteriosis is a manifestation of this imbalance (Uphoff, 2004). Additionally, numerous other potential stressors of striped bass exist in Chesapeake Bay, including loss of deep coldwater refuges due to summer anoxia (Coutant, 1985), spawning stress, and pollutants. Furthermore, studies have demonstrated that striped bass may be particularly susceptible to *M. marinum* relative to other fish species (Wolf & Smith, 1999).

Compounding the difficulties of determining the causative factors of mycobacteriosis in Chesapeake Bay striped bass is the presence of multiple potential etiologic agents. In addition to the classic fish pathogen *M. marinum* and the presumptively nonpathogenic *M. gordonae*, at least 8 additional phenotypically distinct groups of *Mycobacterium* spp. have been isolated from striped bass, some of which may in turn contain multiple species. By far the most common isolate is the newly described slow-growing species *M. shottsii* (Rhodes et al. 2001; 2003), followed by isolates resembling *M. triplex*, *M. scrofulaceum*, *M. interjectum*, *M. simiae*, and *M. szulgai*.
The relative contribution of these individual *Mycobacterium* spp. to overall disease of striped bass in Chesapeake Bay remains unknown.

**Pathogenesis of mycobacteria**

The majority of available information about the pathobiology of mycobacteria derives from studies performed in mammalian systems. Literature on the primary innate immune response to pathogenic mycobacteria in mammals will be briefly reviewed in this section.

**Host cells**

Pathogenic mycobacteria survive and replicate in the macrophages or phagocytic cells of their host organisms. In mammals, monocytic phagocytes are the most commonly colonized cells, although neutrophils and dendritic cells may also internalize mycobacteria (N'Diaye et al., 1998; Demangel & Britton, 2000). *M. leprae*, the causative agent of leprosy in humans, also has the ability to invade fibroblasts and muscle cells (Evans & Levy, 1972). By becoming intracellular, mycobacteria may be shielded from attack by the immune system, especially the humoral arm, and thus have certain advantages over extracellular bacteria. The ability of mycobacteria to reside intracellularly within phagocytes without being killed has been the subject of an enormous amount of research.
Degradative mechanisms of phagocytic cells – phagolysosomal fusion

When a bacterium or other large particle contacts the plasma membrane of a phagocytic cell, the membrane invaginates to create a phagosome containing the foreign body. According the “kiss and run” model proposed by Desjardins (1995), phagosomes are progressively remodeled after formation by interaction with a series of endosomal compartments. Shortly after formation, phagosomes acquire early endosomal markers such as mannose-6-phosphate receptor (M6PR), the inactive pro form of the acid hydrolase cathepsin D (procathepsin D), and the GTPase Rab5. These markers are subsequently lost and phagosomes acquire lysosome-associated membrane glycoproteins (LAMPs), active cathepsin D, and the GTPase Rab7 (Desjardins et al., 1994a; Desjardins et al., 1997). The Rab proteins are thought to play a central role in fusion events between different cellular components in eukaryotic cells (Hall, 1990). Phagosomes are also progressively acidified through the action of endosome-delivered vesicular ATP-ase proton pumps (Mellman et al., 1986; Desjardins et al., 1994b), which can drive the internal pH as low as 4.5 (Rous, 1924; Sprick, 1956; DeDuve et al., 1978) as compared to the surrounding physiologic pH ~7.2 of the cytoplasm. After acidification and maturation, phagosomes fuse with lysosomes, at which point they are called phagolysosomes, or secondary lysosomes. Hydrolytic lysosomal enzymes act in conjunction with the acidic environment of the phagolysosome to break down ingested material.
Avoidance of intracellular killing - mycobacterial strategies

The ability of *Mycobacterium* spp. to evade intracellular killing mechanisms of professional phagocytes has been extensively studied. Numerous mechanisms accounting for this ability have been elucidated, the earliest and best represented in the literature being inhibition of phagosome-lysosome (P-L) fusion. This phenomenon was first described ultrastructurally in 1971 for *M. tuberculosis* (Armstrong & Hart, 1971), and has since been demonstrated with *M. avium* (Frehel et al., 1986b), *M. marinum* (El-Etr et al., 2001), and several other *Mycobacterium* species. The mechanism of P-L fusion inhibition has been debated since its initial description. Various agents produced by intracellular mycobacteria have been implicated, including ammonia (Gordon et al., 1980), various polyanions (Geisow et al., 1980), and anionic sulfatides (Goren et al., 1976). Using a pH-sensitive ultrastructural marker, Crowle et. al. (1991) demonstrated that vesicles containing live *M. tuberculosis* and *M. avium* fail to acidify, whereas those containing heat-killed bacteria acidified normally and were fused by lysosomes. This finding was later corroborated and demonstrated to be the result of exclusion of vesicular proton-ATPase in phagosomes containing live mycobacteria (Sturgill-Koszycki et al., 1994).

A number of recent studies have demonstrated exclusion of proton-ATPase and resultant lack of phagosomal acidification may be just one component of an overall mycobacteria-induced retardation of the phagosomal maturation process. Phagosomes containing mycobacteria do interact with components of the early endosomal network, as evidenced by the presence of plasma membrane glycosphingolipids, transferrin, Rab5,
and procathepsin D (Clemens & Horwitz, 1995; Clemens, 1996; Russell et al., 1996; Clemens et al., 2000b). Late endosomal markers such as proton ATPase and M6PR, however are excluded (Xu et al., 1994; Clemens et al., 2000a). While some studies have shown the late endosomal/lysosomal marker LAMP-1 to be largely absent from mycobacterial phagosomes (Clemens & Horwitz, 1995), other studies have shown it to be present (Sturgill-Koszycki et al., 1994; Xu et al., 1994). This apparent discrepancy may be explained, however, by studies demonstrating that this glycoprotein may be delivered to lysosomes from early endosomes (Rohrer et al., 1996). These observations have led to the current paradigm that pathogenic mycobacteria in mammals persist intracellularly by inhibiting the maturation of their phagosomes to the secondary lysosomal state.

Exactly how mycobacteria manage to inhibit maturation of their phagosome remains unknown. Recent studies have suggested that tryptophan-aspartate-containing coat protein (TACO) may play a role in maintenance of the nonfusiogenic mycobacterial phagosome. TACO, or mouse coronin I, is a plasma membrane protein which has been shown to be crucial to normal phagocytosis (Maniak et al., 1995). TACO is typically only transiently associated with phagosomes, however, it was shown to be stably associated with live *M. bovis* BCG phagosomes throughout a course of infection in mouse cells. Killed BCG phagosomes, in contrast, did not retain TACO (Ferrari et al., 1999). Live BCG in TACO-negative liver Kupffer cells were contained in LAMP-positive vacuoles, a fact which the authors cited as evidence of lysosomal fusion, although as noted above, LAMP may not unambiguously identify lysosomes. Expression of TACO cDNA in nonmacrophage cells promoted bacterial uptake relative to controls, but did not
appear to significantly influence intracellular survival rate. Although these findings are suggestive that TACO may play a role in inhibition of phagolysosomal fusion, they do not explain why mycobacterial phagosomes retain fusiogenicity to earlier endosomal compartments. Adding to the uncertainty of the role of TACO in inhibition of phagolysosomal fusion is a recent study that demonstrated replication of *M. marinum* within the slime mold *Dictyostelium discoideum* in the absence of host coronin (Solomon et al., 2003).

Modulation of cytoplasmic calcium \([\text{Ca}^{2+}]_c\) has also been implicated in disruption of phagolysosomal fusion. An increase in \([\text{Ca}^{2+}]_c\) was observed after phagocytosis of zymosan and killed *M. tuberculosis*, whereas live *M. tuberculosis* did not provoke this increase. Treatment of cells with calcium ionophore caused increased \([\text{Ca}^{2+}]_c\) levels which were accompanied by increased levels of lysosomal fusion and reduced bacterial viability (Malik et al., 2000). Subsequent studies demonstrated that phagosomes containing live *M. tuberculosis* had decreased levels of the calcium-dependent effector proteins calmodulin and CaMKII. Calcium ionophore treatment increased recruitment of these proteins to the mycobacterial phagosome, however, inhibitors of these proteins suppressed the enhanced phagolysosomal fusion seen after ionophore treatment (Malik et al., 2001).

The natural-resistance-associated macrophage protein 1 (*Nramp1*) encoded by the *bec* gene has been implicated in resistance to intracellular pathogens in mammals. Transgenic null allele (*Nramp 1−/−*) mice were developed that lacked normal resistance to the intracellular parasites *Mycobacterium bovis, Salmonella typhimurium*, and
Leishmania donovani. Transfection of these mice with wild-type Nramp alleles restored resistance (Govoni et al., 1996). Nramp1 in mammals localizes to late endocytic/lysosomal compartments of mouse macrophages and is recruited to the membrane of phagosomes containing latex beads (Gruenheid & Gros, 2000). The function of Nramp in the phagosomal membrane, however, remains unclear. While some researchers suggest that Nramp1 is a pH-dependent antiporter that accumulates metals within phagosomes (Blackwell et al., 2001; Goswami et al., 2001), other groups have indicated that Nramp1 is a symporter which exports divalent cations from the phagosome to the cytosol (Jabado et al., 2000). Recently, an Nramp from striped bass, MsNramp, was isolated and sequenced. This Nramp had high sequence similarity to mammalian Nramp2, and was strongly induced in peritoneal exudate cells of striped bass in vivo after intraperitoneal injection of live M. marinum (Burge et al., 2004).

A recent study has demonstrated that production of a serine/threonine protein kinase G by mycobacteria within phagosomes prevented maturation of phagosomes to the secondary lysosomal state and promoted survival of intracellular mycobacteria (Walburger et al. 2004). M. bovis BCG lacking the pknG gene resided in LAMP-positive phagosomes, whereas phagosomes containing wild-type M. bovis remained largely LAMP-negative. PknG transformants of M. smegmatis, which does not express pknG, inhibited fusion of LAMP-containing vacuoles relative to the wild-type. Finally, gene disruption or chemical inhibition of protein kinase G resulted in localization of bacilli to LAMP-positive vacuoles and intracellular killing.
Although inhibition of phagolysosomal fusion appears to be common among pathogenic mycobacteria, several exceptions to this pattern have been reported. *M. lepraemurium*, which is pathogenic in mice, does not appear to significantly inhibit P-L fusion, instead being apparently resistant to lysis by lysosomal enzymes (Hart & Armstrong, 1972; Hart et al., 1987). Lysosomal fusion with phagosomes containing multiple species and strains of mycobacteria including *M. tuberculosis* was observed in mouse J774 macrophages, regardless of whether bacteria were live or killed (McDonough et al., 1993). The authors of this study additionally indicated that mycobacteria escaped from phagosomes into the cytoplasm or unfused compartments. This capability has also been attributed to *M. marinum* (Stamm et al., 2003) but remains controversial.

Lysosomal fusion with phagosomes containing intact mycobacteria has also been described in primary cultures of human alveolar macrophages (Borelli et al., 2002) and mouse peritoneal macrophages (Mor, 1985), and within chronic granulomas of frogs in vivo (Bouley et al., 2001). Other studies have suggested that inhibition of phagosomal maturation and/or phagolysosomal fusion may not be crucial to intracellular survival of mycobacteria. Armstrong et al. (1975) demonstrated lysosomal fusion of phagosomes containing *M. tuberculosis* pre-treated with immune or non-immune rabbit serum. Growth of *M. tuberculosis* in fused vacuoles did not appear to be inhibited. In coinfected mouse macrophages, *M. tuberculosis* and *M. avium* colocalized with *Coxiella burnetii*, the causative agent of Q fever, in an acidified vacuole. While the replication of *M. tuberculosis* within this vacuole was inhibited, the growth of *M. avium* was not (Gomes et al., 1999). Other studies, however, demonstrated that activation of macrophages with
IFN-γ and LPS increased rates of phagolysosomal fusion and negatively impacted bacterial survival (Schaible et al., 1998; Via et al., 1998).

These and other apparently conflicting reports from the mycobacterial literature are illustrative of the complexity of the mycobacteria-host cell interaction. The difficulty in dissecting the host-mycobacteria relationship is likely further compounded by variation between experimental models. Further, it should be noted that differences in intracellular behavior \textit{in vitro} and \textit{in vivo} have been found for a number of intracellular pathogens including \textit{Listeria}, \textit{Cryptococcus}, and \textit{Legionella} (Bhardwaj et al., 1998; Feldmesser et al., 2000; Sturgill-Koszycki & Swanson, 2000). In the end, it is logical to postulate that mycobacteria have not one, but several strategies for survival within host cells, including inhibition of phagosomal maturation/lysosomal fusion and resistance to digestion in fused phagolysosomes.
GOALS AND OBJECTIVES

The overall goal of these studies was to examine the pathobiology of *Mycobacterium* spp. in Chesapeake Bay striped bass (*Morone saxatilis*) via exposure studies and *in vitro* assays. Individual portions of this work were designed to address the following objectives:

Chapter 1.

1. Describe the long-term (~1 year) histological progression of disease in striped bass experimentally infected with *M. marinum*, *M. shottsii*, or *M. gordonae*.
2. Quantify splenic density of injected *Mycobacterium* spp. over the course of infection.
3. Fulfill Koch’s postulates for individual *Mycobacterium* spp. by production of disease and re-isolation of the injected organism.

Chapter 2.

1. Describe the ultrastructural progression of the *M. marinum* granuloma in striped bass.
2. Examine the possibility of alternate, non-acid-fast forms of *M. marinum* within striped bass granulomas.

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3. Describe the early (<72 hour) response of peritoneal exudate cells to injected *M. marinum*.

Chapter 3.

1. Ultrastructurally describe the intracellular behavior of *M. marinum* within striped bass macrophages *in vitro*.
2. Using ultrastructural lysosomal markers, determine if live *M. marinum* inhibits phagolysosomal fusion relative to heat-killed *M. marinum*.
CHAPTER 1

Experimental Mycobacteriosis in Striped Bass (*Morone saxatilis*)

Published:

Striped bass (Morone saxatilis), were injected intraperitoneally with approximately $10^5$ Mycobacterium marinum, M. shottsii sp. nov., or M. gordonae. Infected fish were maintained in a flow-through fresh water system at 18°-21°C, and were examined histologically and bacteriologically at 2, 4, 6, 8, 17, 26, 36 and 45 weeks post-infection. M. marinum caused acute peritonitis, followed by extensive granuloma development in the mesenteries, spleen and anterior kidney. Granulomas in these tissues underwent a temporal progression of distinct morphological stages, culminating in well-circumscribed lesions surrounded by normal or healing tissue. Mycobacteria were cultured in high numbers from splenic tissue at all times post-infection. Standard Ziehl-Neelsen staining, however, did not demonstrate acid-fast rods in most early inflammatory foci and granulomas. Large numbers of acid-fast rods were present in granulomas beginning at 8 weeks post-infection. Between 26 and 45 weeks post-infection, reactivation of disease was observed in some fish, with disintegration of granulomas, renewed inflammation, and elevated splenic bacterial densities approaching $10^9$ colony-forming units g$^{-1}$. Infection with M. shottsii or M. gordonae did not produce severe pathology. Mild peritonitis was followed by granuloma formation in the mesenteries, but, with one exception, granulomas were not observed in the spleen or
anterior kidney. *M. shottsii* and *M. gordonae* both established persistent infections in the spleen, but were present at densities at least two orders of magnitude less than *M. marinum* at all time points observed. Granulomas in the mesenteries of *M. shottsii-* and *M. gordonae-*infected fish resolved over time, and no reactivation of disease was observed.
INTRODUCTION

Mycobacterial infections are common in wild and captive fish stocks worldwide. *Mycobacterium marinum, M. fortuitum,* and *M. chelonae* are the most frequently reported isolates, but other species including *M. neoaurum, M. simiae, M. poriferae* and *M. scrofulaceum* have been cultured from infected fish (Backman et al., 1990; Landsdell et al., 1993; Tortoli et al., 1996; Bruno et al., 1998). Clinical signs of mycobacteriosis are nonspecific and may include scale loss, dermal ulceration, emaciation, exophthalmia, pigmentation changes, and spinal defects (Nigrelli & Vogel, 1963; Gomez et al., 1993; Bruno et al., 1998). Miliary granulomatous inflammation throughout the visera is characteristic of mycobacteriosis, and enlargement of affected organs may occur (Colorni, 1992). Histologically, granulomas resemble those found in mammalian mycobacterial infections, and acid-fast bacilli are usually present (Nigrelli & Vogel, 1963).

The striped bass, or rockfish (*Morone saxatilis*) is among the numerous commercially or recreationally important fish species that are affected by mycobacteriosis. Outbreaks of mycobacteriosis have been described in wild and cultured populations of west coast striped bass, with prevalences reaching 68 and 80%, respectively (Sakanari et al., 1983; Hedrick et al., 1987). *Mycobacterium marinum* was cultured from fish in the latter study, and has also been isolated from wild Pacific striped bass (Landsdell et al., 1993). Recently, visceral and dermal lesions in striped bass from the
Chesapeake Bay and its tributaries were shown to be associated with mycobacterial infection (Vogelbein et al., 1999). An epizootiological study of striped bass from Virginia tributaries of the Chesapeake Bay found up to 62.7% prevalence of mycobacteriosis, based on histological presence of characteristic granulomas. This suggests the disease has significant effects on wild stocks (Cardinal, 2001).

In addition to *Mycobacterium marinum* and several other *Mycobacterium* spp., two recently described species of mycobacteria, 'M. chesapeaki' (Heckert et al., 2001) and *M. shottsii* (Rhodes et al., 2001; 2003), have been isolated from Chesapeake Bay striped bass. Mycobacteria were cultured from splenic tissue of approximately 76% of striped bass that were collected from the Chesapeake Bay or its tributaries and sampled bacteriologically. *M. shottsii* was the most frequently isolated, occurring in more than 70% of culture-positive samples and often reaching densities of $10^4$-$10^6$ colony-forming units per gram (CFU g$^{-1}$) splenic tissue (Rhodes, unpub. data). Lesions in fish from which *M. shottsii* have been cultured, however, have varied widely in severity, and clinical signs of disease were not always present. This has also been true of fish that cultured positive for *M. marinum* and other mycobacteria. Further, co-infections of two or more mycobacterial species frequently occurred. Therefore, controlled experimental infections of striped bass were performed to elucidate the role of selected individual *Mycobacterium* spp. in production of disease.

The temporal progression of experimentally induced mycobacterial disease has been examined in a number of fish species, including plaice (*Pleuronectes platessa*), seabass (*Dicentrarchus labrax*), Atlantic salmon (*Salmo salar*), and goldfish (*Carassius*...
Acute mycobacteriosis with rapid mortality (≤ 8 days) has been produced in striped bass via intramuscular injection of relatively high doses of *Mycobacterium marinum* (Wolf & Smith, 1999). As mycobacteriosis is generally considered to be a chronic disease of fish, pathology produced over long time periods by sublethal doses is relevant to understanding the disease process. To our knowledge, no studies have yet addressed the long-term pathogenesis of mycobacteria in striped bass.

In this study, we experimentally produced chronic infections in striped bass with the mycobacterial species *Mycobacterium shottsii, M. marinum,* and *M. gordonae.* *M. gordonae,* a common environmental isolate, is generally considered to be a non-pathogenic saprophyte, although cases of infection in humans have been reported (McIntyre et al., 1987; Lessnau et al., 1993) The lesions produced by the three mycobacterial species, as well as splenic bacterial density, were examined and compared at regular intervals over a 45-week time period.
MATERIALS AND METHODS

Fish maintenance

Striped bass used in these experiments were obtained as fry from Harrison Lake National Fish Hatchery (Charles City, VA). Fish were maintained within an isolation facility in 1000 l circular tanks supplied with 9 l minute⁻¹ (daily total volume replacements = 13) of heated spring water that was degassed and oxygenated to saturation prior to use. Illumination of the tanks was provided by a combination of fluorescent and natural lighting with photoperiod of the fluorescent lighting adjusted to match local conditions. Fish were maintained on a dry pellet diet and were treated in-tank with 1% sodium chloride (w/v) to alleviate stress each time they were handled. Effluent from tanks containing fish injected with mycobacteria was passed through a 1400 l circular tank containing naïve fish in order to detect horizontal waterborne transmission of the bacteria. Prior to release from the facility, all tank effluents were treated for a minimum contact time of 20 minutes with hypochlorite maintained at a diluted final concentration of 100 mg l⁻¹. Prior to use in the studies, striped bass were maintained under the conditions described above except that the tanks were located separate from the isolation facility and were illuminated with fluorescent light only. Mean initial weights (g) of fish in long- and short-term portions of this study were (mean ± SE) 113.1 ± 2.4 (n = 360) and 126.8 ± 3.4 (n = 168), respectively.
**Mycobacteria**

*Mycobacterium marinum* and *M. shottsii* used in these studies were isolated from splenic tissue of Chesapeake Bay striped bass. *M. gordonae* was isolated from a skin lesion of a Chesapeake Bay striped bass. Isolates were identified using traditional growth and biochemical tests (Kent & Kubica, 1985; Lévy-Frébault & Portaels, 1992). In addition, *M. shottsii* was characterized by antimicrobial sensitivity testing, HPLC mycolic acid analysis and 16S rRNA gene sequencing (Rhodes et al., 2001; 2003). *M. marinum* and *M. shottsii* were passed once intraperitoneally in striped bass and recovered from spleen homogenates by plating on Middlebrook 7H10 agar with OADC enrichment and 0.5% glycerol (MDA). The inoculum of *M. gordonae* was obtained from an archived isolate maintained on MDA. Colonies from the primary isolation plates (*M. marinum, M. shottsii*) or archived culture (*M. gordonae*) were inoculated into Middlebrook 7H9 medium with OADC enrichment and 0.05% polyoxyethylene sorbitan monooleate (Tween 80) (MDB) and incubated at 23°C (*M. shottsii*) or 30°C (*M. gordonae, M. marinum*). *M. gordonae* and *M. marinum* prefer the higher growth temperature of 30°C, whereas *M. shottsii* exhibits optimal growth at the lower temperature (Rhodes et al., 2001). Suspensions of actively growing cultures, as determined turbidimetrically, were pelleted by centrifugation at 12,000 x g for 20 minutes and washed once in Butterfield's phosphate buffer (Anonymous, 1995) with 0.05% Tween 80 (PB). Washed cultures were resuspended in buffer, vortexed vigorously with glass beads (~500 μm diameter) for 2 minutes and filtered through Whatman No. 1 paper to reduce clumping and obtain a
homogeneous suspension. Absorbance at 590 nm was adjusted with PB to 0.05, or approximately $10^7$ CFU ml$^{-1}$, and diluted tenfold in PB prior to injection.

**Infection**

At study initiation, striped bass were anesthetized using 100 mg l$^{-1}$ Finquel (MS-222, Argent Chemical), weighed, and injected intraperitoneally with 100 µl of diluted mycobacterial suspension. In the long-term study, the dose (CFU g$^{-1}$ total body weight) of *Mycobacterium marinum*, *M. gordonae*, and *M. shottsii* delivered was (mean ± SE, n = 89 fish for each treatment) 2300 ± 100, 1400 ± 70, and 1500 ± 90 respectively. The dose of *M. marinum* (n = 40 fish), *M. gordonae* (n = 40 fish), and *M. shottsii* (n = 10 fish) delivered in the short-term study was (mean ± SE) 8700 ± 200, 3100 ± 80, and 17000 ± 400 respectively. The number of sham (PB) injected fish in the long- and short-term experiments was 89 and 40, respectively.

**Sampling interval**

Fish used for the short-term (Group 1) and long-term (Group 2) portions of the study were maintained in separate tanks. The long-term experiment was initiated 10 weeks prior to the short-term experiment. At each sampling, bass were sacrificed using a lethal dose of Finquel and weighed. Group 2 striped bass were sampled (n = 10 unless otherwise noted) 8, 17, 26, 36 (*Mycobacterium gordonae* n = 3), and 45 weeks (*M. gordonae* n = 0, *M. marinum* n = 7) post-inoculation. Group 1 samples (*M. marinum* and *M. gordonae* n=5, *M. shottsii* n=3) were obtained biweekly beginning at week two (*M.
*marinum* and *M. gordonae*) or week 4 (*M. shottsii*) and ending at week 8 post-inoculation. For the first 26 weeks of the long-term experiment and the entire short-term experiment, water temperature was maintained at 18°C. Water temperature increased to 21°C four days after the week 26 sampling of Group 2 and was maintained at this level for the remainder of the study. The change in temperature was not planned and resulted from a switch to back-up heating following the failure of the primary system.

**Bacteriology**

Fish sacrificed at selected exposure intervals were aseptically necropsied. A portion of splenic tissue weighing 0.01-0.5 g was removed, weighed and transferred to a sterile Ten Broeck tissue grinder. Tissue was homogenized in 1.5 ml PB and tenfold serially diluted. Duplicate MDA plates were spread-plated with 0.2 ml homogenate or dilutions thereof. Brain heart infusion agar plates were inoculated with homogenate and monitored for non-acid-fast bacterial contamination. Plates were incubated and examined at 6-8 weeks with final observations made at 3 months. Representative colonies from each sample were acid-fast stained using the Ziehl-Neelsen method. Isolates from more than one-half of samples positive for acid-fast colonies were biochemically characterized to confirm the identity of the isolate. Tests for phenotypic verification included pigmentation, Tween 80 hydrolysis, and production of arylsulfatase, nitrate reductase, niacin, pyrazinamidase, and urease (Kent & Kubic, 1985; Lévy-Frébault & Portaels, 1992).
Data analysis

Splenic mycobacterial densities are expressed as CFU g⁻¹, and means are calculated from log-transformed data. Values preceded by "<" indicate that at least one of the replicate samples was below the detection limit, and values preceded by ">"> indicate that colonies at the highest dilution plated were too numerous to count. Values below or above the detection level were entered as absolute values based on the lowest level of sensitivity or the highest dilution plated. All statistical analyses were performed with Statview (SAS Institute, Cary, NC). Regression analysis of mycobacterial densities and time was performed using simple linear regression and the regression ANOVA table. Differences between infected groups were evaluated using the Friedman nonparametric "two-way analysis of variance by ranks test" and the Mann-Whitney U-test. The Kendall rank correlation test was used to determine if mycobacterial densities correlated with maturation of host response.

Histology

Fish tissues were fixed in 10% phosphate-buffered formalin for at least 72 hours prior to processing. Samples of anterior kidney, liver, spleen, mesenteries, hindgut, and, occasionally, body wall were processed routinely for paraffin histology (Prophet et al., 1994). Samples of body wall were decalcified prior to processing. Sections were cut at 5 μm and stained with hematoxylin and eosin (HE). Selected slides were also stained with the Ziehl-Neelsen acid-fast stain or Ziehl-Neelsen preceded by a 24-hour incubation in 10% periodic acid (HIO₄) (Nyka & O'Neill, 1970). Hereafter, the former technique
will be abbreviated "ZN," and the latter will be abbreviated "P-ZN." All slides were examined on an Olympus AX-70 light microscope.

**Lesion staging**

HE-stained tissues were examined for the presence of granulomas, which were defined as lesions containing epithelioid cells. Fish not displaying lesions with epithelioid cells were classified as negative (-), although pre-granulomatous inflammatory foci may have been present. When granulomas were present, serial sections were stained with ZN or P-ZN to visualize acid-fast bacilli. A staging system was developed based on major morphologic characteristics of observed granulomas (Table 1). Fish displaying granulomas were assigned to one of four lesion stages, depending on the most advanced lesion present. Interpretation of the temporal progression of lesion development was based on the stages present relative to time elapsed post-infection (Table 2).
Table 1. Major histological features of lesion stages. Acid-fast staining described is unmodified Ziehl-Neelsen.
<table>
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<th>Lesion Stage</th>
<th>Major Features</th>
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<tr>
<td>Inflammatory focus/none (-)</td>
<td>Either no lesions, or lesions composed of loosely organized inflammatory cells are present. Few- to no acid-fast bacilli are observed within inflammatory foci.</td>
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<td>Epithelioid granuloma (I)</td>
<td>Lesion is composed of centralized epithelioid cells surrounded by inflammatory cells. Eosinophilic cellular debris may be present in lesion center. Few to no acid-fast bacilli are present.</td>
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<td>Spindle-cell granuloma (II)</td>
<td>Distinct necrotic core is separated from surrounding epithelioid cells by one or more layers of flattened, highly eosinophilic spindle-shaped cells. Few to no acid fast bacilli are present.</td>
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<td>Bacillary granuloma (III)</td>
<td>Numerous acid-fast bacilli are present in core and spindle cell layers. Spindle cell layers are variable in thickness and may be incomplete.</td>
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<td>Recrudescent lesion (IV)</td>
<td>Organization of granuloma is disrupted. Lesion cores are composed of intensely eosinophilic cells, foamy eosinophilic debris, or cells with pyknotic nuclei. Margins of lesion are indistinct, and a wide margin of inflammatory cells may be present. Acid-fast bacilli are absent, sparsely dispersed or present in clumps. Large lesions may replace majority of normal parenchyma in affected organs.</td>
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Table 2. Histological progression of granulomatous inflammation in mycobacteria-infected fish. Fish were assigned to a lesion stage category based on the most advanced lesion observed in sections. The number of fish having reached a particular lesion stage at each sampling point is presented. Stage descriptions are given in the text. Tissue types are abbreviated: Anterior kidney = AK, Spleen = SPL, Mesentery = MES. Short-term and long-term experiments are separated by the bold line.
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*a*Spleens from these two fish were not available for histology

*b*ns = not sampled

*c*One sample was lost before histology could be performed

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RESULTS

Gross pathology

External clinical signs observed in this study were nonspecific and could not be attributed to mycobacterial infection. Sampled fish in all groups including sham-injected controls were occasionally anorexic and/or dark in color. Minor petechiae were observed on the venter of some infected fish, but grossly hemorrhagic skin ulcers similar to those seen on wild caught fish (Rhodes et al., 2001) were not present.

Nodular red foci were grossly visible in the mesenteries of both groups of *Mycobacterium marinum*-infected fish at all time points. These foci appeared to reach peak intensity at week 8 (Group 1) and week 17 (Group 2), at which point the entirety of the visceral fat was red, hardened, and fused into a solid mass. Numerous adhesions of this mass to the body wall were observed at these time points. Multiple red foci were observed on the surface of the liver, and the spleen became progressively darkened and friable, with gray nodules. Buff-colored or gray nodules were also present in the anterior kidney. Grossly visible inflammation of the viscera declined at 26 and 36 weeks, whereas the visceral mass again appeared severely inflamed in fish sampled at 45 weeks. Gross pathology in *Mycobacterium gordoneae* - and *M. shottsii*-infected fish was considerably less severe than that seen with *M. marinum*. Occasional red, nodular lesions were
observed scattered among the visceral fat and mesenteries, but otherwise all internal organs appeared normal. Internal lesions were not seen in sham-injected fish.

**Histopathology**

Granulomatous lesions in fish infected with *Mycobacterium marinum* progressed through a series of morphologically distinct stages (Table 1). Lesion cell types and organization were histologically identical in spleen and anterior kidney, and differed in minor aspects from the mesenteries. Fish were assigned to a stage category based on the most advanced lesion observed, however, multiple granuloma stages were frequently present in individual fish.

*Inflammatory focus (-).* Inflammatory foci in the mesenteries were readily distinguishable from normal parenchyma. Minor foci could be found in most fish examined, regardless of infection status. The structure of pre-granulomatous inflammatory foci, especially in *Mycobacterium marinum*-infected fish, however, differed from that of sham-injected fish. Small inflammatory foci, observed in both infected and, less frequently, in uninfected controls, were composed of loosely organized aggregations of macrophages, lymphocytes, and granulocytes. Macrophages and lymphocytes were rounded with basophilic cytoplasm and small, condensed nuclei. In fish injected with mycobacteria, inflammatory foci were greatly enlarged. A loose nodular organization was present in larger foci, and the interior of the lesion was composed of a branching network of basophilic cells (Figure 1). Small, loosely organized basophilic cells were present around the periphery of this network, and eosinophilic granule cells (EGCs) were
Figure 1. Pre-granulomatous inflammatory focus in mesentery of *Mycobacterium marinum*-infected striped bass. H&E stain, scale bar = 200 μm.
variably present. Acid-fast rods measuring approximately 4 μm in length were observed in small numbers by P-ZN staining, but were rarely observed by ZN. Inflammatory foci in spleen and anterior kidney were not readily distinguishable from normal parenchyma.

**Epithelioid granuloma (I).** Epithelioid cells were tightly packed in the granuloma interior, contained homogeneous, slightly eosinophilic cytoplasm, and had enlarged nuclei with marginalized heterochromatin. Vacuolization was occasionally observed. Larger epithelioid granulomas typically had a central region of intensely eosinophilic cellular debris containing pyknotic and karyorrhectic nuclei (Figure 2a). Epithelioid granulomas in the spleen and anterior kidney were generally surrounded by a thin margin of basophilic cells, while a more extensive matrix of inflammatory cells surrounded mesenteric lesions. Acid-fast bacilli were rarely observed by ZN staining within epithelioid granulomas (Figure 2b), although light, diffuse staining was occasionally present. P-ZN staining revealed varying numbers of bacilli, which were found in clumped aggregations within the necrotic core or singly in the surrounding epithelioid cells. These bacilli were polymorphic, ranging in length from 1μm to 4μm, and beaded forms were present.

**Spindle cell granuloma (II).** Spindle cell granulomas were characterized by the presence of spindle-shaped cells with intensely eosinophilic cytoplasm and condensed nuclei. These cells formed a band that separated a distinct necrotic core from surrounding epithelioid layers (Figure 2c), and appeared to be formed by flattening and elongation of the innermost layers of epithelioid cells. At later times post-infection, stage II granulomas typically had thick (>5 cells) spindle cell layers and enlarged necrotic cores.

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Figure 2a-d. Progression of granuloma morphology in spleen of *Mycobacterium marinum*-infected striped bass. Left column is stained by H&E, while right column is ZN stain of a close serial section. a,b) Epithelioid granuloma with centralized eosinophilic debris. c,d) Spindle-cell granuloma with well demarcated epithelioid and spindle-cell layers. Scale bars = 100 μm.
with ceroid pigments. The presence and staining properties of acid-fast bacilli in spindle cell granulomas were similar to that of the epithelioid stage (Figure 2d). In the anterior kidney and spleen, spindle cell granulomas were typically single, roughly spherical and circumscribed by a layer of flattened basophilic cells and/or connective tissue. Tissue parenchyma external to the granulomas appeared normal in organization and composition. In contrast, spindle cell granulomas in the mesenteries were surrounded by large margins of inflammatory cells and connective tissue. Frequently, adjacent granulomas appeared to fuse, creating large multinodular lesions surrounded by contiguous layers of spindle and epithelioid cells (Figure 3a). Large numbers of EGCs were often present in the inflammatory tissue surrounding mesenteric spindle cell granulomas.

*Bacillary granuloma (III).* Bacillary granulomas were morphologically similar to spindle cell granulomas by HE, with the exceptions that epithelioid layers were somewhat reduced in thickness, and more ceroid pigment was present in the core (Figure 2e). Granular basophilic material was also occasionally visible in the core debris. Both ZN and P-ZN techniques stained large numbers of individual, ~4 μm acid-fast bacilli. Bacilli were found in the core as well as intracellularly in the spindle cell layers (Figure 2f). The diffuse acid-fast staining previously observed by ZN was absent. Bacillary granulomas in all tissues were typically well demarcated by connective tissue capsules. In contrast to the extensive inflammatory tissue present around spindle cell granulomas in the mesenteries, the tissue surrounding bacillary granulomas showed evidence of healing and regeneration (Figure 3b). Localized areas of inflammation were still present, and
Figure 2e-h. Progression of granuloma morphology in spleen of *Mycobacterium marinum*-infected striped bass. Left column is stained by H&E, while right column is ZN stain of a close serial section. e,f) Bacillary granuloma with individual acid-fast bacilli in core and spindle-cell layers. g,h) Bacillary granuloma with attenuated spindle-cell layers, lightly staining necrotic core, and acid-fast bacilli. Scale bars = 100 μm.
Figure 3. *Mycobacterium marinum* granulomas in mesenteries of striped bass. a) Large, coalescing spindle-cell granulomas, with widespread inflammation and fibrosis in surrounding tissue (8 weeks p.i.). b) Bacillary granulomas surrounded by largely normal mesenteric tissue (26 weeks p.i.). H&E stain, scale bars = 200 μm.
typically contained high numbers of EGCs. At later times post-infection, bacillary granulomas with attenuated spindle cell layers and lightly staining cores were commonly observed (Figure 2g). In these lesions, the spindle cell layers were reduced to one or two cell thicknesses or were absent, with the core abutting directly on epithelioid cells. Acid-fast bacilli were demonstrable by ZN and P-ZN in the lesion core, as well as in areas where identifiable spindle cells remained (Figure 2h). Acid-fast bacilli were rarely observed in surrounding epithelioid or normal tissue. The integrity of the epithelioid layers and surrounding connective tissue appeared to be maintained in all bacillary granulomas, but lesions with attenuated spindle cell layers were generally more polymorphic than earlier lesions.

Recrudescent lesion (IV). Disintegration of bacillary granulomas and widespread reappearance of inflammation in *Mycobacterium marinum*-infected fish was observed beginning at 26 weeks post-infection. Bacillary lesions of all forms, including those with thick spindle cell layers, were observed in the process of disintegration. In smaller recrudescent lesions, spindle cell layers were absent or disrupted, and the necrotic material of the core was replaced with amorphous, intensely eosinophilic cells containing condensed nuclei, similar to those seen in epithelioid granulomas (Figure 4a). A wide, amorphous border of epithelioid and inflammatory cells surrounded the lesions. Larger recrudescent lesions often displaced considerable areas of the parenchyma in affected tissues (Figure 4b). The core of these lesions was highly expanded and typically composed of foamy, eosinophilic debris, although a cellular core with pyknotic and karyorrhectic nuclei was occasionally present. Fluid-filled, cystic lesions were also
Figure 4. Progression of recrudescent lesions in *M. marinum*-infected fish. a) Bacillary granulomas and small recrudescent lesion (mesentery, 26 weeks). Integrity of spindle cell and epithelioid layers is compromised, necrotic core material is replaced with eosinophilic cell debris, and inflammation is renewed around the lesion. Scale bar = 200 μm. b) Advanced recrudescent lesion (spleen, 36 weeks). Lesion containing core of foamy eosinophilic material occupies a large portion of the organ. Scale bar = 1 mm. c) Advanced recrudescent lesion (anterior kidney, 45 weeks). Entire parenchyma of pronephros is replaced with nodular organizations of inflammatory tissue and fibrosis. Scale bar = 200 μm. H&E stain.
observed. In some cases, the disorganized remnant of a bacillary granuloma containing many acid-fast bacilli was observed within recrudescent lesions. Both ZN and P-ZN techniques stained acid-fast bacteria in recrudescent lesions, although the latter typically revealed larger numbers of bacteria. In some cases, large areas of recrudescent lesions were acid-fast-negative.

In one of seven *Mycobacterium marinum*-infected fish at 45 weeks post-infection, the entirety of the anterior kidney and the majority of the spleen were replaced by nodular organizations of loosely packed inflammatory cells and extensive fibrosis (Figure 4c). Both ZN and P-ZN stains revealed large numbers of acid-fast bacilli within the nodules and in the surrounding connective tissue. Grossly, the anterior kidney was enlarged and composed entirely of buff colored nodules, and the posterior kidney had completely degenerated. The spleen was largely composed of gray nodules and was friable. Similar lesions were observed in a second *M. marinum*-infected fish at 45 weeks, but lesions did not occupy as much of the affected organs.

Granulomas in fish infected with either *Mycobacterium shottsii* or *M. gordonae* were similar in morphology and progression to those of *M. marinum*-infected fish. These lesions, however, were smaller and greatly reduced in number. With the exception of one spindle cell granuloma in the anterior kidney of a single *M. shottsii*-infected fish, granulomas were restricted to the mesenteries. Also, the degree of inflammation observed in the mesenteries of *M. shottsii*- or *M. gordonae*-infected fish did not approach that seen with *M. marinum*. Bacillary granulomas were observed in only one *M. shottsii*-
and two *M. gordonae*-infected fish throughout the course of the study, and recrudescent lesions did not occur.

Granulomas were observed in one sham-injected fish in both groups 1 and 2 at the eight week sample and from one sham-injected fish obtained from group 2 at week 17. The single granuloma in the Group 1 fish was acid-fast-negative and resembled parasitic granulomas observed in wild-caught striped bass. Well-developed spindle cell granulomas were present in the liver of the Group 2 fish at week 8. These granulomas had acid-fast interiors, although bacilli were not visible. No mycobacteria were isolated from the spleen of this fish. One small, ZN-negative, poorly organized granuloma was observed in the anterior kidney of the 17-week fish, and no mycobacteria were recovered from the spleen.

Granulomatous inflammation due to *Mycobacterium marinum* progressed in a similar manner in all affected organs (Table 2). Epithelioid and spindle cell granulomas were present in the mesenteries of infected fish at the first sampling (2 weeks). In both spleen and anterior kidney, epithelioid granulomas were present at 4 weeks, and spindle cell granulomas at 6 weeks. At 8 weeks, bacillary granulomas were present in mesenteries of one fish from both experimental groups, in the spleen of two fish from Group 1, and in the anterior kidney of one fish from Group 2. From 17 weeks onward, the majority of fish had bacillary granulomas in all three tissues. Recrudescent lesions were observed in anterior kidney, spleen, and mesenteries of a single fish at 26 weeks, and in all organs of at least two sampled fish thereafter.
In *Mycobacterium shottsii*-infected fish, mesenteric spindle cell granulomas were not observed until eight weeks, and bacillary granulomas were present in one fish at 36 weeks post-infection. With the exception of Group 1 fish at eight weeks, granulomas were present in a minority of fish sampled (Table 2).

Granuloma progression in *Mycobacterium gordonae*-infected fish was similar to that seen with *M. shottsii*. Spindle cell granulomas were observed at 8 weeks in Group 1, and at 17 weeks in Group 2 fish. Interestingly, small bacillary granulomas were present in both of the *M. gordonae*-infected fish examined histologically at week 36.

In general, the morphology and progression of granulomas associated with the liver was similar to that seen in other organs. The majority of these granulomas were located in the mesenteries surrounding the liver, among exocrine pancreatic cells, or surrounding blood vessels passing through the organ. Most granulomas directly involving the parenchyma appeared to have originated peripherally and eventually incorporated liver tissue. Therefore, it was difficult to ascertain which, if any, granulomas had arisen entirely within the liver parenchyma. Bacillary granulomas and recrudescent lesions did significantly impact liver tissue at later times post-infection with *M. marinum*.

**Bacteriology**

Acid-fast colonies were selected from 101 of 175 culture-positive fish, isolated, and examined phenotypically. All isolates recovered were confirmed to be mycobacterial species injected at the beginning of the study, with one exception. In this instance,
co-infection with a second mycobacterium was detected with CFU g⁻¹ one order of magnitude lower that the injected isolate, *Mycobacterium shottsii*. Acid-fast colonies from sham samples (n=42 fish) were observed on 6 occasions but were phenotypically different from the three injected isolates. No granulomas were observed in the tissues of these fish. One of 7 uninjected fish in the tank exposed to effluent from other holding tanks was positive for *M. marinum*, containing $3.7 \times 10^5$ CFU g⁻¹ splenic tissue. Epithelioid granulomas were observed in the anterior kidney and liver, but not the spleen or mesenteries, of this fish.

Splenic densities of the three mycobacterial species in infected fish over the course of this study are shown in Figures 5 (short-term experiment) and 6 (long-term experiment). Mean *Mycobacterium marinum* concentrations increased approximately one order of magnitude between 4 and 8 weeks in Group 1, whereas mean *M. shottsii* and *M. gordonae* densities decreased by greater than one order of magnitude. In Group 2, mean densities of *M. marinum* reached $\sim 10^9$ CFU g⁻¹ at 36 and 45 weeks. Regression analysis revealed a significant relationship (p < 0.0001) between *M. marinum* cell densities and time. Mean densities of *M. shottsii* ranged from $<3.2 \times 10^2$ CFU g⁻¹ to $<1.6 \times 10^4$ CFU g⁻¹, and mean densities of *M. gordonae* ranged from $<1.3 \times 10^3$ CFU g⁻¹ to $>1.3 \times 10^7$ CFU g⁻¹ over the course of the long-term experiment. The high upper figure for the latter is derived from the three *M. gordonae*-infected fish remaining at week 36. From weeks 8 to 26, mean *M. gordonae* densities peaked at $>7.9 \times 10^3$ CFU g⁻¹. Between 8 and 36 weeks in the long-term experiment, splenic mycobacterial densities of inoculated fish differed significantly (p < 0.05) depending on species. Heterogeneity in mycobacterial loads
Figure 5. Box plots of splenic mycobacterial density (colony forming units [CFU] g\(^{-1}\)) vs. time post-injection in the short-term experiment. Horizontal lines, from top to bottom, represent 10th, 25th, 50th (median), 75th, and 90th percentiles.
Figure 6. Box plots of splenic mycobacterial density (CFU g$^{-1}$) vs. time post-injection in the long-term experiment. Horizontal lines, from top to bottom, represent 10th, 25th, 50th (median), 75th, and 90th percentiles.
between fish was characteristic of all groups but was particularly pronounced for *M. gordonae* and *M. shottsii*-injected fish. Mean densities of *M. gordonae* and *M. shottsii* remained significantly lower than *M. marinum* (*p* < 0.006) throughout the long-term experiment with maximum concentrations of approximately 10⁷ and 10⁶ CFU g⁻¹, respectively, occurring in individual fish.

There was no correlation of mycobacterial concentration and lesion stage for any mycobacterial species in the short-term experiment. However, a highly significant correlation (*p* < 0.0002) was found in the long-term experiment between splenic *Mycobacterium marinum* densities and lesion progression (i.e.: -, I, II, III, IV) in the spleen and mesentery. *M. gordonae*-injected fish exhibited significant (*p* = 0.031) correlation between splenic mycobacterial burden and maturation of mesenteric granulomas. No such correlation was found in *M. shottsii*-infected fish.
DISCUSSION

Experimentally induced piscine mycobacteriosis typically involves an initial acute inflammatory response to injected mycobacteria, followed by the development of a chronic granulomatus disease state with low associated mortality (Bruno et al., 1998; Colorni et al., 1998). Using goldfish as an experimental model, Talaat et al. (1998) demonstrated that mean survival time and transition to the chronic granulomatus state was dependent on initial dose of *Mycobacterium marinum*. Acute peritonitis, reduced granuloma formation, and high mortality were observed in fish receiving $10^8$-$10^9$ organisms, whereas fish receiving $10^7$ bacteria survived to the end of the study and exhibited well-developed granulomas. Acute mycobacteriosis with high mortality has also been produced in striped bass by high-dose intramuscular *M. marinum* exposure (Wolf & Smith, 1999). In the present study, injection of *M. marinum* produced acute inflammation in the peritoneal cavity, which abated concomitantly with the development of mature granulomas in all organs. Splenic bacterial load remained roughly constant during the development of mature granulomas between 8 and 17 weeks post exposure. After 17 weeks, however, considerable bacterial replication occurred within granulomas, as reflected by both splenic counts and histological appearance of acid-fast rods. This replication preceded a phase of granuloma disintegration and reappearance of acute, fulminant disease. These results indicate piscine mycobacteriosis may transition between
phases of chronic granuloma formation and acute disseminative disease, in a manner similar to mammalian mycobacterioses such as tuberculosis (Cotran et al., 1999). The cues for this transition in mammals remain poorly understood, but immune suppression of the host is generally recognized as a factor in reactivation disease (Parrish et al., 1998). Whether the recrudescence of mycobacterial disease observed in this study was precipitated directly by the mycobacteria or by an exogenous, immunomodulatory factor is unknown at this time.

The closely related species *Mycobacterium marinum* and *M. shottsii* (Rhodes et al., 2001) produced very different pathology in the striped bass of this study. Both mycobacteria established persistent infections in the spleen, but granulomatous inflammation in spleen and anterior kidney was observed, with one exception, only in *M. marinum*-infected fish. Whereas *M. marinum* produced severe pathology and a secondary phase of reactivation disease, mesenteric inflammation due to *M. shottsii* was considerably less severe, and mesenteric granulomas very rarely contained large numbers of ZN-detectable bacteria. In addition, mean densities of *M. marinum* in the spleen exceeded that of *M. shottsii* by approximately three orders of magnitude at the later time points of the study. Unlike the *M. shottsii*-infected fish in this study, wild-caught fish from which *M. shottsii* is the sole isolate often display severe granulomatous disease (Rhodes, unpub. data). These findings strongly suggest that in the wild, *M. shottsii* can cause disease similar to that produced by *M. marinum* in this study. Under the experimental conditions of this study, *M. shottsii* was able to establish a persistent, latent infection state. Several factors, such as temperature, salinity, or fish stress may be
involved in the activation of these infections in wild fish. These factors may also be
involved in the production of skin lesions in wild-caught fish (Vogelbein et al., 1999;
Rhodes et al., 2001), which were not observed after injection with any mycobacterial
species in this study.

In *Mycobacterium marinum*-infected fish, the transition from spindle cell
granulomas to bacillary granulomas and, in one fish, recrudescence lesions had already
begun in all organs before the water temperature shift from 18°C to 21°C occurred.
Therefore, it is unlikely that elevated temperature was solely responsible for the observed
progression of disease. It is possible, however, that the elevated temperature accelerated
the growth of *M. marinum* and exacerbated the pathology seen at 36 and 45 weeks. *M.
gordonae*- and *M. shottsii*-infected fish did not exhibit bacillary granulomas until 36
weeks, after the temperature shift. Only one *M. shottsii* fish developed these lesions and
only two *M. gordonae*-infected fish were sampled histologically after 26 weeks, however,
so no substantive evidence was found that the temperature shift was responsible for
disease progression.

The putatively non-pathogenic *Mycobacterium gordonae* was persistent in the
spleens of striped bass throughout the course of the study and produced mesenteric
granulomas identical in form to *M. shottsii*. Bacillary granulomas in *M.
gordonae*-infected fish at 36 weeks suggest that this mycobacterium is capable of survival
for long periods of time in the striped bass host. Another presumably non-pathogenic
mycobacterium, *M. smegmatis*, has also been shown to be pathogenic in fish (Talaat et
al., 1999). These results indicate that so-called "environmental isolates" such as *M.
gordonae* may play a role in mycobacterial disease of wild or aquacultured fish stocks.

During development of granulomas to the spindle cell stage, acid-fast bacilli were rarely demonstrated by the commonly used Ziehl-Neelsen technique. Truant's fluorescent acid-fast stain and Taylor's gram stain were also applied in an attempt to demonstrate cryptic mycobacteria, without improvement over ZN. Pretreatment with periodic acid, however, revealed mycobacteria in serial sections that were negative by ZN. This technique has been shown to increase staining of chromophoric forms of *Mycobacterium tuberculosis* and *M. leprae* in tissue sections and smears (Harada, 1977; Nyka & O'Neill, 1970), as well as *M. marinum* in broth culture (Dhople, 1985). Although the existence of acid-fast and non-acid-fast mycobacteria has long been recognized, the chemical basis for this variation remains unknown, and the clinical significance of chromophoric forms is still debated. Several researchers have suggested the acid-fastness of mycobacteria may be dependent on their replicative or physiologic state, with bacteria in log phase being more chromophobic than those in stationary phase (Dhople, 1985; Reich, 1971). In this study, however, the presence of bacillary granulomas correlated with a nearly 80-fold increase in mean splenic CFU g⁻¹, suggesting actively replicating *M. marinum* were not chromophobic. In fish that had not yet developed bacillary granulomas, nearly all bacilli present within lesions appeared to be chromophobic, but large numbers of mycobacteria were cultured. These observations indicate viable chromophoric forms of *M. marinum* were present within granulomas of infected striped bass. It is possible that these forms
represent a resting state, and are capable of reversion to their original acid-fast form given appropriate conditions or stimuli.

Demonstration of acid-fast, unbranched rods in tissue sections or smears is considered diagnostic for *Mycobacterium* spp. in fish (van Duijn, 1981). As demonstrated here, however, mycobacteria present in early granulomas may not stain by unmodified ZN. Mycobacterial lesions with few or no acid-fast bacilli have also been described at early time points in experimentally infected seabass (Colorni et al., 1998) and in wild yellow perch *Perca flavescens* (Daoust et al., 1989). In the latter study, the use of Fite's acid-fast stain improved detection of acid-fast bacilli. Therefore, the type of stain used, as well as the stage of mycobacterial infection, may strongly influence the detection of acid-fast bacilli. This indicates caution should be used in the interpretation of acid-fast-stained tissues from fish, and that mycobacterial infection should not be ruled out on the basis of a negative ZN stain in fish displaying granulomas.

Detection of mycobacteria by culturing can also be problematic. Recovery of mycobacteria from granulomatous lesions can be precluded by the use of harsh disinfectants, inappropriate culture conditions (e.g.: media, temperature, duration of incubation) and the fastidiousness of the causative agent (Shotts & Teska, 1989; Rhodes et al., 2003). Generally, no attempt is made to perform quantitative analyses of the mycobacterial burden in infected tissues. In the present study, the need to reduce contaminating bacteria with chemical agents was avoided with careful aseptic necropsy. Cultural conditions were also maximized to enhance recovery by using a mildly selective medium (MDA) and incubating at an environmentally relevant temperature for an
extended time period. Combining these cultural methods with a quantitative assay enabled assessments of mycobacterial densities during disease progression.

Spindle cell and bacillary granulomas observed in this study were similar to those described in wild and aquacultured striped bass (Sakanari et al., 1983; Hedrick et al., 1987), as well as wild Chesapeake Bay fish (Vogelbein, unpub. data). Large numbers of acid-fast bacilli were demonstrated by unmodified ZN in mycobacterial granulomas by Hedrick et al. (1987) and Sakanari et al. (1983), suggesting these fish may have been in a similar state of disease as fish with bacillary granulomas in this study. It is not known, however, how long bacillary granulomas remain intact within striped bass, or if the presence of bacillary granulomas necessarily leads to recrudescent disease. The progression of disease in experimentally infected striped bass may eventually be used to interpret histological findings in wild fish, but the influence of several environmental factors on this progression must first be examined before such parallels can be drawn.

In order to assess the potential for horizontal waterborne transmission of mycobacteria, uninjected striped bass were maintained in a tank receiving effluent water from all other tanks containing infected fish. One of 7 fish sampled from this tank at week 45 had epithelioid granulomas in the anterior kidney and liver, while the spleen and mesentery were free of inflammation. *Mycobacterium marinum* was isolated from the spleen of this fish at a density of $3.7 \times 10^5$ CFU g$^{-1}$. This indicates *M. marinum* is shed by infected fish, and that fish may obtain infections from the water. Further studies will be necessary to confirm this finding.
CHAPTER 2

Ultrastructure of *Mycobacterium marinum* Granuloma in Striped Bass (*Morone saxatilis*)

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MANUSCRIPT ABSTRACT

An emerging epizootic of mycobacteriosis currently threatens striped bass 
(Morone saxatilis) populations in Chesapeake Bay, USA. Several species of 
mycobacteria, including Mycobacterium marinum, species resembling M. avium, M. 
gordonae, M. peregrinum, M. scrofulaceum, and M. terrae, and the new species M. 
shottsii have been isolated from diseased and healthy bass. In this study, we describe the 
ultrastructure of developing M. marinum granulomas in experimentally infected bass over 
a period of 45 weeks. The primary host response to injected mycobacteria was formation 
of large macrophage aggregations containing phagocytosed bacilli. M. marinum were 
always contained within phagosomes. Close association of lysosomes with mycobacterial 
phagosomes, as well as the presence of electron-opaque material within phagosomes 
suggested phagolysosomal fusion. Development of granulomas involved epithelioid 
transformation of macrophages, followed by appearance of central necrosis. 
Desmosomes were present between mature epithelioid cells. The necrotic core region of 
M. marinum granulomas was separated from overlying epithelioid cells by several layers 
of flattened, electron-dense spindle-shaped cells. These cells appeared to be formed by 
compression of epithelioid cells and, aside from a flattened nucleus, did not possess 
recognizable organelles. Following the development of well-defined, paucibacillary 
granulomas, secondary disease was observed. Recrudescence was marked by bacterial
replication followed by disruption of granuloma architecture, including loss of epithelioid and spindle cell layers. In advanced recrudescent lesions, normal tissue was replaced by macrophages, fibroblasts, and other inflammatory leukocytes. Large numbers of mycobacteria were observed, both intracellular and suspended in cellular debris.
INTRODUCTION

Granulomas are chronic inflammatory lesions composed of various cell types, including macrophages, lymphocytes, fibroblasts, and granulocytes. Granuloma morphology varies widely depending on eliciting agent, host species, and other factors, but lesions are typically organized in concentric cellular layers, with a central region of macrophages surrounded by a collar of various other inflammatory cells. Macrophages in the interior of the granuloma differentiate into characteristic forms known as epithelioid (epithelial-like) cells (Sutton & Weiss, 1966; Papadimitriou & Spector, 1971). Epithelioid cells appear by hematoxylin and eosin (HE) staining as polygonal cells with large, pale nuclei and eosinophilic, faintly granular cytoplasm. These cells are usually tightly apposed with indistinct cytoplasmic boundaries, forming a solid mass of tissue.

Granulomas are most frequently elicited by agents that are resistant to intracellular degradative processes (Stenger et al., 1967; Papadimitriou & Spector, 1972; Black & Epstein, 1974; Doenhoff, 1997). There are two major types of granulomas, depending on the eliciting agent. Foreign body granulomas are formed in response to inert particles, such as talc or material from surgical sutures (Cotran et al., 1999). Typically, foreign body granulomas are composed of a solid mass of epithelioid cells and giant cells, with the eliciting agent often visible in the center. The other major granuloma type, the immune granuloma, is formed in response to insoluble particles that stimulate a
cell-mediated immune response, such as mycobacteria. Cytokines produced by T cells, such as IL-12 and interferon-γ, play an important role in formation and maintenance of immune granulomas (Cooper et al., 1993; Ehlers et al., 2000). One prominent feature of immune granulomas produced by mycobacteria such as *Mycobacterium tuberculosis* is caseous necrosis in the lesion core, which produces a highly acidic, anoxic environment that may serve to degrade otherwise refractory bacilli.

The structure and development of mycobacterial granulomas in mammals, especially those produced in response to *Mycobacterium tuberculosis* and BCG (attenuated *M. bovis*), have been described (Papadimitriou & Spector, 1972; Adams 1976; Browett et al., 1979). Mycobacterial granulomas in poikilotherms are less well understood, as few controlled temporal morphological studies have been performed. In light of recent interest in *M. marinum* infections of fish as model systems for exploring mycobacterial pathogenesis (Talaat et al., 1998; Talaat et al., 1999; Davis et al., 2002), detailed study of the structure and development of teleost granulomas is necessary.

In a previous study (Gauthier et al., 2003), we described the histologic progression of experimental mycobacterial disease in striped bass over a period of 45 weeks. Lesions produced in response to injected *Mycobacterium marinum* displayed a consistent morphological progression from early inflammatory cell aggregations to well-developed epithelioid granulomas with necrotic cores. In addition, disintegration of granulomas and subsequent secondary disease was observed. In this study, we examine this morphological progression at the ultrastructural level.
MATERIALS AND METHODS

The present work used tissues collected concomitantly with histologic and bacteriologic samples in a previous laboratory exposure study. Detailed methods of fish maintenance, mycobacterial infection, and sampling interval are given in Gauthier et al. (2003). Briefly, striped bass *Morone saxatilis* (n=130, wt. [mean ± SE] 116.7 ± 3.9 g) were infected intraperitoneally with $2.6 \times 10^5$ (long-term study, 8-45 weeks) or $1.1 \times 10^6$ (short-term study, 2-8 weeks) *Mycobacterium marinum*. Fish were sacrificed at 2, 4, 6, 8, 17, 26, 36, and 45 weeks post-injection (p.i.) for histologic and bacteriologic sampling. Samples of spleen, mesentery, and anterior kidney were taken for ultrastructural examination from three fish at each sampling point.

**Early time-point samples**

In a third study, fish were injected with $1.4 \times 10^6$ *Mycobacterium marinum* as described previously (Gauthier et al. 2003). At 24 and 72 hours p.i., fish were lavaged for peritoneal cells. Ten ml ice-cold L-15 (Liebovitz) medium with 100U/ml sodium heparin (Sigma, St. Louis, MO) was injected into the peritoneal cavity and withdrawn through a ventral incision ten minutes later. Cells were centrifuged at 400 x g for 10 minutes, washed once in L-15, and fixed as described below.
Processing for electron microscopy

Tissues from fish sampled between 2 and 45 weeks post-infection were fixed for 4-6 hours in ice-cold 2% glutaraldehyde/2.5% paraformaldehyde/0.15M sucrose/0.1 M sodium cacodylate buffer (pH 7.2), followed by 1 hour postfixation in 1% OsO₄/0.1M sodium cacodylate. Cells obtained by peritoneal lavage were fixed for 30 minutes in cold 1.5% glutaraldehyde/2% paraformaldehyde/0.15M sucrose/0.1M sodium cacodylate (pH 7.2), and postfixed as described for tissue samples. Dehydration of both tissues and peritoneal cells was accomplished by a graded ethanol series with a 1 hour en bloc staining with saturated uranyl acetate in 70% ethanol. Alcohol was replaced by several changes of propylene oxide, after which tissues were infiltrated and embedded in Spurr's resin (Electron Microscopy Sciences, Ft. Washington, PA). Ultrathin (90 nm) sections were cut on a Reichert-Jung ultramicrotome, mounted on carbon-stabilized Formvar-coated copper one-hole grids, stained with Reynold's lead citrate (Reynolds 1963) for 7 minutes and examined on a Zeiss CEM 902 transmission electron microscope operating at 80kV.
RESULTS

Cell types, organization, and ultrastructural features were similar in granulomas from spleen, anterior kidney and mesenteries, and were consistent in all fish sampled. Descriptions given below apply to developing granulomas in all three tissues except where specifically noted. Throughout the text, the term "macrophage aggregation" is used to describe the early inflammatory response to mycobacteria. This term should not be confused with "macrophage aggregate," which is a focal accumulation of pigment-bearing macrophages in the spleen, anterior kidney, or liver of many teleost fish (Wolke, 1992).

Early macrophage aggregation (24-72 hours, lavage material)

Large, roughly spherical aggregations of macrophages were observed in lavaged peritoneal cells preparations at 24 and 72 hours after injection with mycobacteria (Figure 1a). The peripheral cytoplasm of aggregated cells formed long, slender filopodia that interdigitated with adjacent cells, while non-aggregated macrophages typically had shorter and broader pseudopodia. Organelle morphology of cells within aggregations and non-aggregated cells was similar at both times. Aggregated macrophages were
Figure 1. a) Aggregated macrophages. Bar = 10 μm. b) Aggregated macrophages with phagocytosed mycobacteria (arrows) and eosinophilic granule cell (egc). Bar = 1 μm. c) Transverse section of mycobacterium (m) within phagolysosome. Space between phagolysosomal membrane (arrowheads) and bacterial cell wall (arrow) is filled with electron-opaque material. Phagolysosome is surrounded by electron-opaque lysosomes. Bar = 0.5 μm. d) Macrophage with electron-opaque phagosome (arrow) containing debris and a bacillus (arrowhead). Note extensive filopodial interdigitations between adjacent cells. Bar = 5 μm. e) Dead cell (*) being phagocytosed by adjacent macrophage(s). Bar = 1 μm. f) Electron-lucent phagosome (*) containing debris and mycobacterium (arrowhead). Bar = 1 μm. (all peritoneal lavage, 24 hours post-infection).
polymorphic, ranging from 5-7 μm in greatest dimension, and had ovoid to lobular nuclei with eccentric nucleoli (Figure 1b). Cytoplasm was finely granular. Organelles included ovoid mitochondria, perinuclear Golgi apparatus, rough endoplasmic reticulum (rER), and spherical to ovoid membrane-limited endosomes of varying electron opacity.

Endosomes were observed to fuse with phagosomes containing ingested material, and are thus collectively referred to as lysosomes, although their morphological variation suggests a degree of functional and/or developmental heterogeneity. Phagosomes contained cellular debris, myelin figures, and/or mycobacteria.

All intracellular mycobacteria were contained within a limiting phagosomal membrane. Typically, phagosomes were tightly conformant to bacilli (Figures 1b,c), but expanded phagosomes containing debris and mycobacteria were also observed (Figures 1d,f). Electron-opaque lysosomes were frequently observed in close proximity to mycobacterial phagosomes, and electron-opaque material was often present in the space between the mycobacterial cell wall and phagosomal membrane (Figure 1c). Cell death was observed frequently within macrophage aggregations, and dead cells were phagocytosed by other macrophages (Figure 1e). When dead cells containing one or more bacilli were phagocytosed, expanded, electron-lucent phagosomes containing mycobacteria and debris resulted (Figure 1f).

Non-aggregated peritoneal exudate cells included macrophages, eosinophilic granule cells (EGCs), neutrophilic granule cells (NGCs) and lymphocytes. EGCs were readily identifiable by large, homogeneous electron-opaque granules, and neutrophils were differentiated from morphologically similar macrophages by the presence of
characteristic striated granules. These features and cell types were morphologically consistent with previous reports (Bodammer, 1986; Bodammer & Robohm, 1996). Mycobacteria were rarely observed within non-aggregated macrophages. Occasional granulocytes were observed within macrophage aggregations (Figure 1b).

**Inflammatory focus/Epithelioid granuloma (2-8 weeks)**

The macrophage component of early mesenteric inflammatory foci was morphologically similar to macrophage aggregations from lavage samples. In addition to these cells, granulocytes and lymphocytes were present within and surrounding inflammatory foci. Large quantities of extracellular collagen microfibrils were observed within and surrounding large mesenteric inflammatory foci at 2 weeks post-infection. Collagen was always accompanied by cells with dense cytoplasm, lobular to crenulate nuclei, extensive swollen rER, and few mitochondria. These cells were putatively identified as fibroblasts based on their consistent association with extracellular collagen and highly synthetic appearance. Fibroblast cell shape was variable, with both rounded and highly elongate forms observed.

Early macrophage aggregations rapidly developed into organized, stratified epithelioid granulomas. Stratified granulomas were present in the mesenteries as early as 2 weeks, and in all three sampled tissues at 6 weeks. Three morphologically distinct cellular layers could be seen in organized lesions: 1) epithelioid cell layer, 2) spindle cell layer, and 3) necrotic core (Figure 2a). A transitional layer of compressed epithelioid cells between 1) and 2) was occasionally present.
Figure 2. a) Granuloma stratification. Epithelioid (1), compressed epithelioid (1a), spindle-cell (2), and core layers (3) are visible. Bar = 5 μm (spleen, 8 weeks p.i.). b) Epithelioid macrophages. Multiple desmosomes (arrowheads) join adjacent cells. Bar = 1 μm. (anterior kidney, 17 weeks p.i.). c) Desmosomes between epithelioid cells. Tonofilaments (arrows) are joined to electron-opaque attachment plaques via short perpendicular filaments. Bar = 0.5 μm. (spleen, 8 weeks p.i.). d) Spindle cells. Note short fimbrial interdigitations (arrow, inset). Bars = 1 μm, 0.5 μm inset. (mesentery, 8 weeks p.i.). e) Crystalloid inclusions (arrowheads) within epithelioid cell. Bar = 0.5 μm. (spleen, 8 weeks p.i.). f) Granuloma boundary. Epithelioid cells (lower right) are separated from splenic parenchyma (upper left) by fibrous tissue (*). Bar = 1 μm. (spleen, 8 weeks p.i.).
Epithelioid cells first appeared at the center of inflammatory foci, and constituted the majority of cells in the interior of developing granulomas. Intermediate morphological forms between macrophages and epithelioid cells were commonly observed in early lesions, with cells becoming increasingly epithelioid in appearance toward the lesion center. Epithelioid cells formed the outer layers of granulomas with core necrosis. Ultrastructurally, epithelioid cells resembled macrophages with increased cytoplasmic and nuclear volume, and increased numbers of mitochondria and rER profiles. These cells were highly compressed, and cytoplasmic boundaries were indistinct (Figure 2b). Bundles of intermediate filaments could often be observed in the cytoplasm of epithelioid cells. Unlike macrophages of earlier aggregations, epithelioid cells rarely contained phagosomes or mycobacteria. In addition to cytoplasmic interdigitations, numerous desmosomes (macula adherens) were formed between adjacent epithelioid cells (Figures 2b,c). Desmosomes varied in length, typically ranging between 100-400 nm, but single desmosomes of up to 1.2 μm were observed. Typical desmosomal components of attachment plaques and tonofilaments were present, however, the structures were somewhat unusual in that cytoplasmic intermediate filaments generally ran parallel to desmosomes and were linked to attachment plaques by short perpendicular filaments. In addition, a dense intermediate line was not typically observed, as is typical in mammalian desmosomes (Ghadially, 1988).

Spindle-cell layers appeared concomitantly with core necrosis. Histologically these cells were highly compressed and intensely eosinophilic (Gauthier et al., 2003). Ultrastructurally, spindle cells were highly compressed, with electron-opaque cytoplasm.
and no recognizable organelles aside from flattened, heterochromatic nuclei (Figure 2d). Cellular boundaries were indistinct, and adjacent cells elaborated extensive arrays of short interlocking fimbrial cytoplasmic projections (Figures 2d, inset). In some lesions, the boundary between epithelioid cells and spindle cells was sharply defined, whereas in others, a gradual morphological progression to the spindle-cell state was observed.

Necrotic core regions were composed largely of amorphous, relatively electron-lucent debris with occasional identifiable cell fragments. Ultrastructural observation of mycobacteria corresponded with histologic findings (Gauthier et al., 2003). Bacilli were very rare in core material of granulomas from fish that were negative for acid-fast bacilli by the Ziehl-Neelsen technique.

Crystalloid inclusions were frequently observed within all layers of granulomas, especially at later stages in development (Figure 2e). Crystalloids were irregular, trigonal to hexagonal, measured 0.2-0.7 μm between apices, and had no discernable internal periodicity. In macrophages and early epithelioid cells, crystalloids were generally contained within a phagosome or fine limiting membrane, whereas crystalloids in mature epithelioid cells, spindle cells and core material appeared to be free in the cytoplasm or extracellular. Similar crystalloid structures were observed within intact and degranulating EGCs surrounding granulomas. Crystalloids were highly eosinophilic by hematoxylin and eosin stain.

Granulomas were typically surrounded by a thick capsular matrix of collagen microfibrils. In early epithelioid granulomas, EGCs, lymphocytes, and macrophages of varying epithelioid character were present throughout the deposited collagen. These cells
were somewhat polymorphic and often conformed tightly to the surrounding matrix. The cellularity of the collagenous layer generally decreased with the development of core necrosis and spindle cells, so that in well-stratified granulomas the outer layers of epithelioid cells were separated from normal tissue parenchyma by an acellular fibrous matrix (Figure 2f).

**Bacillary granuloma/Recrudescence**

When present in large numbers within intact granulomas, bacteria were located within core and spindle cell layers. Core bacilli were suspended in necrotic material. Due to extreme compression and lack of distinct limiting membranes, it could not be definitively determined if bacilli in the spindle cell layer were intra- or extra-cellular (Figure 3a). Transverse division of individual bacilli was commonly observed, and large concentrations of bacteria were often seen at the interface between spindle cells and necrotic core material. Both intact mycobacteria and structures suggestive of degrading bacilli were observed in core and spindle cell layers (Figure 3b).

Disruption of bacillary granulomas was marked by a loss of lesion stratification, disappearance of spindle cell layers, a ragged outer margin of epithelioid cells, and reappearance of inflammatory cells on the lesion periphery (Figure 4a). Rather than the compressed, tightly joined concentric layers observed in earlier granulomas, epithelioid cells were oriented in several different directions and not as tightly apposed. Toward the center of lesions, cells had relatively electron-opaque cytoplasm and few
Figure 3. a) Mycobacteria within necrotic material (n) and spindle cell layer (s) of granuloma. Bar = 5 μm. (mesentery, 36 weeks p.i.). b) Intact (arrows) and possible degenerating (arrowhead) *M. marinum* in core material. Electron transparent zone surrounding mycobacteria is likely artifactual. Bar = 0.5 μm. (anterior kidney, 17 weeks p.i.).
Figure 4. a) Disrupted granuloma. Note ragged edge of epithelioid layer (arrows) and absence of lesion zonation. Bar = 10 μm. b) Lesion interior. Cells with epithelioid appearance (e) and cells with morphology intermediate between epithelioid and spindle cells (I) are present. Electron-opaque granular deposits are present between cells (arrow). Bar = 1 μm. c) Lesion margin. Matrix of collagen microfibrils (cf) is populated with fibroblasts (arrows) and macrophages (arrowheads) of varying electron-opacity. Lipid droplets are common in macrophages. Bar = 1 μm. (all mesentery, 32 weeks p.i.).

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organelles. Intercellular pockets of granular, electron-opaque material were frequently observed (Figure 4b). Necrotic material and numerous mycobacteria were observed in the core regions of disrupted lesions. The fibrous capsule of disrupted lesions was often highly cellular, with macrophages, granulocytes, and fibroblasts (Figure 4c).

Secondary disease was marked by replacement of granulomas with a poorly organized, loose reticular network of macrophages, lymphocytes, fibroblasts, and granulocytes. Epithelioid cells were rare or absent in these lesions. Intracellular mycobacteria in intact macrophages were contained within both tightly conformant and expanded, debris-containing phagosomes (Figure 5a). Transverse division of bacteria within phagosomes was commonly observed. Large areas of dead cells with bacilli were present in some lesions, as were concentrations of debris-swollen macrophages (Figures 5b,c,d). A residual membrane was frequently observed around bacilli suspended in cellular debris (Figure 5c). Granuloma remnants in varying degrees of disintegration were occasionally observed (Figure 5e). The interior of these remnants was typically replaced with macrophages and other inflammatory leukocytes, as well as diffuse necrotic material.
Figure 5.  a) Advanced recrudescent lesion. Granuloma organization is lost and normal tissue is replaced with inflammatory leukocytes and/or dead cells. Mycobacteria (arrows) are present within both conformant and expanded, debris-containing phagosomes. Bar = 1 μm.  b) Intact mycobacteria (arrows) among cellular remnants. Bar = 10 μm.  c) Detail of mycobacteria in cellular debris. Note presence of crystals (arrow) and residual membrane. Bar = 1 μm.  d) Debris-replete and degrading macrophages. Bar = 1 μm.  e) Granuloma remnant. Fibrous/cellular shell remains (arrows), but epithelioid cells of interior have been replaced with loose organization of inflammatory leukocytes and necrotic material. Bar = 10 μm.  (all anterior kidney, 45 weeks p.i., except e) spleen, 45 weeks p.i.).

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DISCUSSION

Histologic development of granulomas in *Mycobacterium marinum*-infected striped bass has been described previously (Gauthier et al., 2003). This study expands those findings to the ultrastructural level, and provides information on the very early (24-72 hour) cellular response to injected mycobacteria. Formation of the *M. marinum* granuloma in striped bass *Morone saxatilis* appeared to be effected primarily by macrophages, which were capable of considerable polymorphism in various lesion layers. Lymphocytes, fibroblasts, and granulocytes were also present at all stages of granuloma development. Membrane contact between these cells and macrophages was common, and macrophages often contained products apparently derived from EGCs (i.e.: crystalloids). These observations suggest a role for accessory cells in the formation and maintenance of granulomas in fish.

Intracellular *Mycobacterium marinum* in macrophage aggregations and early epithelioid granulomas were always bounded by a limiting phagosomal membrane. In many cases, mycobacteria appeared to be contained within fused phagolysosomes, based on electron-opaque material within the phagosome and apparent fusion events with surrounding lysosomes (Figure 1c). This observation is surprising, in light of the paradigm that pathogenic mycobacteria, including *M. marinum*, survive intracellularly in *vitro* by inhibiting phagosome-lysosome fusion of host macrophages (Armstrong & Hart,
Morphological evidence of in vivo phagolysosomal fusion has also been presented for long-term M. marinum granulomas in frogs, and it has been suggested that macrophage processing of mycobacteria may differ in vitro and in vivo (Bouley et al., 2001). Preliminary findings by our group, however, indicate that fusion of lysosomes with M. marinum phagosomes also occurs in vitro (data not shown). Additional experiments are currently underway to resolve this issue.

Epithelioid cells, so called because of their histologic resemblance to true epithelial cells, are derived in mammals from the monocyte/macrophage lineage. The transformation of mammalian monocytes into epithelioid cells is well documented in vivo (Papadimitriou & Spector, 1971; Adams, 1974; Browett et al., 1979), and has been demonstrated in vitro for avian monocytes (Sutton & Weiss, 1966). Whether piscine epithelioid cells derive from the same lineage is less clear, especially considering their unusual capability to elaborate desmosomes. Desmosomes are generally considered to be characteristic of true epithelial or mesothelial cells (Ghadially, 1988), although desmosome-like structures have been observed between histiocytes in certain human dermatopathies, as well as rat carrageenan granuloma (Caputo & Gianotti, 1979; Monis & Valentich, 1986). Noga et al. also observed the presence of desmosomes in piscine granulomatous inflammation, and postulated that either 1) the capacity of macrophages to elaborate desmosomes is a primitive characteristic that has been lost in avian and mammalian orders, or 2) the cells involved in certain types of piscine inflammation derive from a non-macrophage lineage (i.e.: mesothelial cells) (Noga et al., 1989). The findings of the present study indicate that the epithelioid cells of Mycobacterium
marinum granulomas in striped bass are derived from macrophages. Macrophages in the majority of granulomas displayed a continuum of morphological features between amoeboid phagocyte and static, mature epithelioid cell, typically with increasing epithelioid characteristics toward the lesion core. Mature epithelioid cells were tightly packed and strongly linked by interdigitations and desmosomes, and migration of dissimilar cell types through epithelioid layers was not observed. This arrangement implies sequential development of epithelioid cells from free macrophages, as well as transformation of epithelioid cells into spindle cells. In addition, we have observed desmosome formation between adjacent adherent peritoneal macrophages \textit{in vitro} (Gauthier et al., 2001). These results strongly suggest that mycobacterial granulomas in fish are, in fact, composed of differentiated macrophages, as in mammals, and that piscine macrophages are capable of desmosome formation. Thus, desmosome formation appears to be a primitive characteristic of macrophages. Whether the human macrophage and the functionally and morphologically analogous cell termed "macrophage" in fish arise from homologous lineage, however, remains to be determined.

Mammalian epithelioid cells in BCG granulomas have reduced phagocytic capacity, but retain pinocytic and excretory capability, the ability to synthesize DNA and RNA, and the ability to divide (Papadimitriou & Spector, 1971; 1972). The maintenance of endosomes, ER, Golgi apparatus, and numerous mitochondria in the epithelioid cells of striped bass granulomas indicates that they may also play an active role in granuloma maintenance and function. Mitotic figures among epithelioid cells or granuloma-associated macrophages were rare histologically and were not observed.
ultrastructurally in the present study. Papadimitriou & Spector (1971) have described epithelioid cell division as asymmetric cytokinesis resulting in an immature-appearing daughter macrophage. No immature-appearing cells were observed among the epithelioid cell layers in this study. These observations indicate that increases in granuloma cell number were due primarily to recruitment and differentiation of cells, rather than division of cells within the lesion. This is consistent with previous findings in rabbits and frogs (Ando & Dannenberg, 1972; Bouley et al., 2001). In epithelioid cells near the lesion core, organelles became less numerous and cytoskeletal elements more prominent, suggesting a transition in role from metabolically active cell to structural sequestering element. Reduced metabolic activity in these cells would not be unexpected, considering that granulomas have no internal vascular elements, thus making cells in the lesion interior dependent on diffusion of oxygen through overlying cellular layers. Spindle cells bordering the lesion core had no recognizable organelles save a highly condensed nucleus, and therefore appeared metabolically inactive. The thickened, fimbrial structures observed between spindle cells differed markedly from the cellular interdigitations in overlying layers, however, suggesting that the transformation from mature epithelioid cells to spindle cells is not simply one of passive compression, but instead involves active formation or rearrangement of cellular elements.

*Mycobacterium marinum* granulomas were paucibacillary by both histologic and ultrastructural observation from early epithelioid through well-organized spindle cell stages. Whether the nidus of intra-granuloma bacterial replication lay within the core material or spindle cells was not determined by this study, although the distribution of
bacteria in some lesions suggests rapid replication at the spindle cell-core interface and subsequent spread throughout both regions (Figure 3a). Structures suggestive of degrading mycobacteria were also frequently observed within these regions (Figure 3b). Death and degradation of mycobacteria in the lesion core would not be surprising, considering that the granuloma interior is generally considered to be hostile to bacterial life due to acidity, hypoxia, and the presence of hydrolytic enzymes. The presence of morphologically intact bacilli and the observed rapid outgrowth of bacteria in bacillary granulomas, however, indicated that a subpopulation of mycobacteria was capable of withstanding the environment of the inner granuloma. The mechanisms by which these few mycobacteria were able to survive, as well as the cues that led to the observed rapid bacterial outgrowth remain unknown at this time. Further study of *M. marinum* infection in striped bass may provide insights into mechanisms of latency and secondary disease for other pathogenic mycobacteria.

Latency of mycobacteria in both endothermic and poikilothermic animals remains poorly understood (Parrish et al., 1998; Flynn & Chan, 2001). It is known that pathogenic mycobacteria (e.g.: *Mycobacterium tuberculosis*) remain viable and infectious in asymptomatic individuals after primary infection (Opie & Aronson, 1927; Feldman & Baggenstoss, 1939). Acid-fast staining frequently fails to reveal mycobacteria in healed primary infection foci, however, leading some to postulate the existence of chromophobic forms of mycobacteria via either alteration or loss of the cell wall (Harada, 1977; Khomenko, 1987). Indeed, varying degrees of acid-fast chromophobicity have been demonstrated for mycobacteria *in situ* and *in vitro* (Nyka & O’Neill, 1970; Dhople 1985),
and we have found evidence for chromophobic mycobacteria within paucibacillary striped bass granulomas (Gauthier et al., 2003). No obvious differences in fine structure were observed for the rare mycobacteria within such lesions however, and no alternate bacillary forms could be discerned. Therefore, this study did not find evidence for the existence of differing forms of mycobacteria within striped bass granulomas. Rather, it appeared the large numbers of mycobacteria in bacillary granulomas arose from a small number of bacteria that survived within the central region of the lesions. No widely accepted ultrastructural description of altered or cell wall-deficient mycobacterial forms in situ exists, however, so the hypotheses of their existence cannot be rejected on the basis of this study. Immunolocalization or in situ hybridization studies will be necessary to more clearly demonstrate the quantity, form, and location of viable bacteria within striped bass granulomas.

Numerous crystalloid eosinophilic inclusions were an unusual feature of the striped bass granuloma. Several types of spontaneously forming eosinophilic crystals have been described in the mammalian literature. Charcot-Leyden crystals (CLCs) are commonly described in human conditions of eosinophilia, such as allergy, parasitic infection, eosinophilic granuloma, and various neoplasms (Dvorak et al., 1990; Carson et al., 1992). CLCs are composed of the enzyme lysophospholipase (lysolecithin acylhydrolase, EC 3.1.1.5) (Weller et al., 1982), which spontaneously forms regular geometric structures, usually hexagonal bipyramids, when released from damaged or degranulating eosinophils (El-Hashimi, 1971). Crystalline inclusions are also found in the core of most intact human eosinophil granules (Miller et al., 1966), although
lysophospholipase has been ultrastructurally localized to a subpopulation of granules without crystalline cores (Dvorak et al., 1988). We have not observed crystalline cores within striped bass EGCs. Eosinophilic crystals of irregular shape isolated from deletional mutant and transgenic mice have been identified as Ym1, a protein of unknown function with \textit{in vitro} chitinase activity (Guo et al., 2000). Crystals of this protein have been postulated to play a role in cell pathology during murine \textit{Cryptococcus neoformans} infections (Feldmesser et al., 2001). Although the crystals associated with striped bass granulomas bear similarities in morphology and origin with the mammalian crystals mentioned, biochemical or immunological characterization will be necessary for definitive identification.
CHAPTER 3

Interaction Between *Mycobacterium marinum* and Macrophages of Striped Bass
(*Morone saxatilis*)
INTRODUCTION

An epizootic of mycobacteriosis currently threatens striped bass (*Morone saxatilis*) stocks in Chesapeake Bay, USA. The disease is characterized by granulomatous inflammation in the viscera, especially spleen and anterior kidney. Ulcerative granulomatous dermal lesions are also observed in some fish. Fish with skin lesions and/or severe visceral disease are often emaciated. Visceral disease prevalence of up to 76% has been recorded in Virginia tributaries of Chesapeake Bay (Cardinal, 2001), and initial estimates of visceral disease prevalence in the Bay mainstem based on large scale trawl surveys range from 37-59% depending on time of year (Gauthier, unpublished data).

The specific etiologic agent of mycobacteriosis in Chesapeake Bay striped bass remains unclear. Several phenotypically distinct mycobacterial isolates have been recovered from both diseased and non-diseased fish (Rhodes et al., 2004), and the majority have not been assessed for pathogenicity in a laboratory setting. The most common individual isolate from Chesapeake Bay bass is *Mycobacterium shottsii*, a newly described, nonchromogenic, slow-growing species (Rhodes et al., 2001; Rhodes et al., 2003), followed by isolates that resemble *M. shottsii* but which are photochromogenic. Other isolates are biochemically similar to species such as *M. triplex*, *M. scrofulaceum*, *M. interjectum*, or *M. szulgai*. *M. marinum*, the most commonly described etiologic
agent of fish mycobacteriosis worldwide, represents relatively few (~3%) of the mycobacterial isolates from Chesapeake Bay bass (Rhodes et al., 2004).

*M. marinum* has been shown to be highly pathogenic in experimentally infected striped bass. Rapidly lethal mycobacteriosis was produced in striped bass after high intramuscular doses of *M. marinum*, and increased disease severity in striped bass relative to tilapia (*Oreochromis* spp.) was demonstrated (Wolf & Smith, 1999). In long-term laboratory exposure studies using sublethal doses of *M. marinum*, striped bass developed progressive visceral disease and a secondary phase of recrudescence (Gauthier et al., 2003). This secondary phase was characterized by disintegration of granulomas, severe disseminated inflammation, and widespread tissue destruction.

The ability to survive and replicate within phagocytic cells is a common feature of pathogenic mycobacteria in a wide range of vertebrate hosts. The interaction between mycobacteria and mammalian phagocytes has been extensively studied, and a general consensus exists that phagosomes containing mycobacteria resist the normal processes of acidification and phagolysosomal fusion (Brown et al., 1969; Armstrong & Hart 1971; Crowle et al., 1991; Sturgill-Koszycki et al., 1994). Further, pathogenic mycobacteria appear to retard the maturation of mammalian phagosomes and their progression to the phagolysosomal state by preventing fusion with components of the late, but not early, endosomal network (Clemens & Horwitz, 1995; Clemens, 1996; Russell, 2001).

Behavior of mycobacteria within macrophages of poikilotherms is less well characterized than in mammals. The first study to examine the ultrastructural interaction between *Mycobacterium* spp. and fish phagocytes was performed with anterior kidney
phagocytes of rainbow trout (*Oncorhynchus mykiss*) (Chen et al., 1998). In more recent study, fusion of thorium dioxide-labeled lysosomes with phagosomes containing *M. marinum* was found to be inhibited relative to phagosomes containing the nonpathogenic *M. smegmatis* (El-Etr et al., 2001). We have found, however, that *M. marinum* phagosomes in striped bass peritoneal macrophages exhibit fusion *in vivo* (Gauthier et al., 2004) and *in vitro* (Gauthier, unpublished data) with membrane-bound electron-opaque organelles morphologically consistent with lysosomes. Similar observations have been made on *M. marinum* within granulomas of frogs (Bouley et al., 2001), and *Mycobacterium* spp. in fish tissues (Chen et al., 1998).

In this study, the ultrastructural interaction between *M. marinum* and macrophages of the striped bass *in vitro* was further examined. Two ultrastructural lysosomal markers were used to assess phagolysosomal fusion: exogenous horseradish peroxidase (HRP) and albumin-coated 10 nm gold beads (Au-BA). HRP is a commonly used endosomal marker which is taken up by mammalian cells both via fluid-phase pinocytosis and mannose/N-acetylglucosamine receptor-mediated endocytosis (Lang & de Chastellier, 1985) and passes through early endosomal compartments before it ultimately localizes in late endosomes/primary lysosomes (Kielland & Cohn, 1980; Marsh et al., 1986; de Chastellier et al., 1995; de Chastellier & Thilo, 1997). Au-BA was also used as a marker of primary and secondary lysosomes, as has been reported previously (Weidner & Sibley, 1985; de Carvalho & de Souza, 1990; de Chastellier & Thilo, 1997). The tendency of metallic particulates such as gold, ferritin, and thorium dioxide to accumulate in lysosomes is well known (Arborgh et al., 1974; Armstrong & Hart, 1975; Ghadially et al., 1977). In
addition to descriptive and semiquantitative marker studies, the killing activity of *M. saxatilis* macrophages vs. intracellular *M. marinum* was quantified via colorimetric bactericidal assay.
MATERIALS AND METHODS

Fish

Striped bass *Morone saxatilis* (360-475 g) were collected by pound net or hook and line from the York River, Virginia. Fish were transferred to 3000 l circular tanks supplied with flow-through sand-filtered York River water at ambient temperature and salinity. At the time these experiments were performed, water temperature was 10°-11°C, and salinity was approximately 13 ppt. Tanks were illuminated by fluorescent lighting matched to seasonal photoperiod. Fish were fed live baitfish (primarily mummichog *Fundulus heteroclitus*) daily and were acclimatized to captivity for a minimum of two weeks before use in experiments.

Cell culture and collection

Striped bass were injected intraperitoneally with 750 µl Freund’s Incomplete Adjuvant 10 days prior to cell collection in order to elicit peritoneal exudate cells. Fish were anesthetized with a lethal dose (100 mg l⁻¹) of tricaine methanesulfonate (Finquel, Argent) immediately prior to cell collection. Anesthetized fish were injected intraperitoneally with 10 ml ice-cold Leibowitz L-15 medium containing 100U ml⁻¹ penicillin + 0.1 mg ml⁻¹ streptomycin (P-S) and 100U ml⁻¹ sodium heparin (pH 7.2). After 10 minutes, lavage fluid containing peritoneal exudate cells (PE) was withdrawn through
a ventral incision. PE were washed twice in ice-cold L-15/2% fetal bovine serum (FBS, Invitrogen)/P-S/10U ml⁻¹ sodium heparin and counted on a Reichert Brightline hemacytometer. Viability of PE as assessed by trypan blue exclusion was greater than 95% for all fish sampled. Cells were resuspended to $8 \times 10^6$ cells ml⁻¹ in ice-cold L-15/0.1% FBS/P-S and added to either 96-well (100 μl well⁻¹; $8 \times 10^5$ cells) or 6-well (1 ml well⁻¹; $8 \times 10^6$ cells) tissue culture plates. Plates were placed in a 12°C incubator and cells were allowed to adhere for 4 hours, at which point medium was replaced with L-15/5% FBS/P-S. Temperature was maintained at 12°C for the remainder of the experiments.

**Bacteria**

*Mycobacterium marinum* (VIMS isolate M30, fish-passaged) was grown and prepared as described previously (Gauthier et al., 2003). Briefly, cultures of *M. marinum* were grown to log-phase in Middlebrook 7H9 medium with OADC enrichment and 0.05% polyoxyethylene sorbitan monooleate (Tween 80) (MDB) at 30°C. Bacteria were pelleted by centrifugation at 12,000 $\times$ g for 20 minutes and washed once in Butterfield’s phosphate buffer (Anonymous, 1995) with 0.05% Tween 80 (PB). Washed cultures were resuspended in 1-2 ml PB, vortexed vigorously with glass beads (~500 μm diameter) for 2 minutes and filtered through Whatman No. 1 paper to remove clumps of bacteria and obtain a homogeneous cell suspension. The viability of mycobacteria processed by this method was determined to be >90% using fluorescent viability stains (*BacLight* LIVE/DEAD kit, Molecular Probes). Absorbance of the mycobacterial suspension was
adjusted to an OD$_{590}$ of 0.15 with Hank's Balance Salt Solution (HBSS), and tenfold dilutions were plated on Middlebrook 7H10 agar with OADC enrichment and 0.5% glycerol (MDA) for subsequent enumeration. Heat-killed *M. marinum* were prepared by heating aliquots of the bacterial suspension to 70°C in a water bath for 2 hours. Sterility was confirmed by plating on MDA. Cultures of *Yersinia ruckeri* (Hagerman strain; National Fish Health Research Laboratory, Leetown, WV, no. 11.40) were grown in Tryptic Soy Broth (TSB) for 24 hours at room temperature. *Y. ruckeri* were killed by suspension in 1% formalin for 24 hours, pelleted, washed four times in HBSS, and adjusted to an OD$_{590}$ of 0.15 (approximately $10^8$ cells ml$^{-1}$ based on previous counts of live OD$_{590}$ 0.15 suspensions). Sterility was confirmed by plating on Tryptic Soy Agar.

**Bactericidal assay**

A quantitative bactericidal assay was performed using a modification of existing methods (Peck, 1985; Chung & Secombes, 1987; Mshana et al., 1998). After 24 hours in antibiotic-containing medium, cells in 96-well plates were washed twice with L-15 (no additives), then overlayed with 150 µl L-15/5% FBS without antibiotics. Fifty µl of bacterial suspension giving an approximate target:effector ratio of 5.7:1 were then added to wells. Control wells received 50 µl HBSS without mycobacteria. One and one-half hours were allowed for settling of bacteria and phagocytosis, at which point cells were washed twice with L-15 and overlayed with 200 µl L-15/5% FBS with no antibiotics. Immediately (Time 0) and at 24 hour time intervals thereafter, medium was removed from one plate and 50 µl sterile 0.1% Tween-20 in distilled water were added to lyse
cells. Lysis was allowed to proceed for 10 minutes, then 150 µl MDB were added to each well. The plate was then incubated at 30°C in a humid chamber for 48 hours to allow bacterial outgrowth. After incubation, 10 µl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) were added to each well and color was allowed to develop for 4 hours at 30°C. After 4 hours, MTT reduction was halted and bacterial cells lysed by addition of 25 µl 10% SDS to each well. Plates were placed on a mechanical rocker overnight to dissolve the purple MTT reduction product and scanned at 590 nm with an automated plate reader (Molecular Devices, Sunnyvale, CA). Killing Index (KI) was calculated by dividing the mean value of quadruplicate wells at time x (T_x) by the mean value of quadruplicate wells at time 0 (T_0). KI values above one indicated bacterial outgrowth while KI below 1 indicated killing. KI was calculated for both raw OD_{590} values (KI_{OD}) and estimated actual bacterial numbers (KI_{act}). The latter values were derived by transforming raw OD_{590} values by a standard curve of known bacterial numbers vs. MTT reduction. To generate the standard curve, a suspension of \textit{M. marinum} was enumerated by plate-counting on MDA, and serial two-fold dilutions were made in quadruplicate in 96-well plates. Plates were incubated for 48 hours at 30°C in 150 µl MDB/50µl lysis buffer, then MTT was added and plates were read spectrophotometrically as described above. A curve was fitted to means of quadruplicate wells with standard deviation <10% of the mean using quadratic least-squares methods (r²=0.98). KI data are presented as mean KI ± SD from 5 fish.
Lysosomal labeling

After incubating adherent cells for 24 hours in antibiotic-containing medium, bovine albumin-coated 10 nm gold beads (Au-BA, Sigma) were added to each well of 6-well TC plates to a final dilution of 1:20. Cells were incubated for 10 hours with Au-BA, then horseradish peroxidase (HRP, Sigma) was added to a final concentration of 0.1 mg ml\(^{-1}\). Controls for endogenous peroxidase did not receive HRP. Two hours after addition of HRP, cells were washed and overlayed with antibiotic-free medium as described above.

Infection and sampling

Immediately after washing, 0.5 ml of bacterial suspension containing live \(M.\) \textit{marinum}, HK \(M.\) \textit{marinum} or FK \(Y.\) \textit{ruckeri} in HBSS was added to each well, giving an approximate target:effector ratio of 5.7:1 for mycobacteria or 6.6:1 for FK \(Y.\) \textit{ruckeri}. Endogenous HRP controls received 0.5 ml live \(M.\) \textit{marinum} or HBSS. One and one half hours were allowed for settling and phagocytosis, after which cells were washed twice with L-15 and overlayed with 2 ml L-15/5% FBS without antibiotics. At 2, 24, 48, and 72 hours, medium was removed, wells were washed once with HBSS, and monolayers were fixed for 30 minutes with room temperature (RT) 1.5% glutaraldehyde/0.1M Na cacodylate/0.15M sucrose (pH 7.2). The number of cells recovered from peritoneal lavage varied between fish and was limiting to the number of timepoints and treatments that could be performed per individual. Sample sizes for 2, 24, 48, and 72 hours were 6, 8, 2, and 1 fish, respectively. Observations were made on cells exposed to FK \(Y.\) \textit{ruckeri}.
at 2 and 24 hours only (n=4). Controls for endogenous peroxidase in *M. marinum*-uninfected (n=6) and –infected (n=2) cells were performed at 24 hours.

**Enzyme cytochemistry**

Peroxidase was demonstrated ultrastructurally by modification of existing methods (Robbins et al. 1971). After fixation, cells were washed three times with 0.1M sodium cacodylate and once with 0.05M Tris (pH 7.6). Cells were then incubated for 30 minutes with 1 mg ml⁻¹ dianaminobenzidine (DAB, Sigma) in 0.05M Tris (pH 7.6). Hydrogen peroxide was added to final concentration of 0.01% and the peroxidase reaction allowed to proceed for 10 minutes. Cells were washed 3 times with 0.1M sodium cacodylate before postfixation.

**Processing for transmission electron microscopy (TEM)**

Cells were postfixed in their tissue culture plates with 1% OsO₄ in 0.1M sodium cacodylate for 1 hour at room temperature. Dehydration was accomplished by a graded ethanol series with a 1 hour *en bloc* staining with saturated uranyl acetate in 70% ethanol. Cells were removed from tissue culture plates with propylene oxide and transferred into microcentrifuge tubes. After two additional changes of propylene oxide, cells were infiltrated and embedded in Spurr's resin (Electron Microscopy Sciences). Ultrathin (90 nm) sections were cut on a ultramicrotome (Reichert-Jung Ultracut E), mounted on either copper 200-mesh grids or one-hole grids coated with carbon-stabilized Formvar, and examined on a Zeiss CEM 902 transmission electron microscope operating at 80kV. To
maintain contrast of the DAB reaction product, sections were not stained with lead citrate.

**Quantification of phagolysosomal fusion**

Quantification of mycobacterial phagosome fusion with peroxidase- and/or Au-BA-positive lysosomes was accomplished by examining 100 mycobacteria-containing phagosomes for each fish/treatment. Phagosomes were considered positive for Au-BA fusion when gold beads were present within the phagosomal membrane. Positive peroxidase reaction was recorded when DAB reaction product was observed within phagosomes or when DAB reaction product-containing lysosomes were observed in an advanced state of fusion with phagosomes, i.e.: when membranes were fused and the lumen of the two compartments was unambiguously contiguous. Au-BA label was not found in all cells, therefore a secondary analysis was performed whereby only cells containing Au-BA were examined for the presence of the label within phagosomes. A minimum of 50 phagosomes per fish/treatment was examined for this analysis.
RESULTS

Adherent cells

The majority of cells in adherent monolayers were ultrastructurally consistent with macrophages from preparations of striped bass peritoneal exudate cells as described previously (Bodammer, 1986; Gauthier et al., 2004). Eosinophilic and neutrophilic granule cells were rare in all preparations. Macrophage nuclei were round to ovoid with largely marginated heterochromatin, and a prominent nucleolus was frequently observed. Pseudopodia and ruffling of the plasmalemma were always present. Organelles included rER and sER profiles, perinuclear Golgi apparatus, and ovoid mitochondria. Large numbers of polymorphic membrane-bound endosomes were observed in the cytoplasm. In unlabeled preparations that were not processed for enzyme cytochemistry, endosomes were variably electron-opaque and occasionally contained small inclusions. Cells were more flattened than those previously observed in nonadherent in vivo preparations (Gauthier et al., 2004), and consequently measured up to 20 μm in greatest dimension.

A small proportion of monolayers from all in vitro fish/treatment preparations consisted of cells morphologically distinct from macrophages. These cells were highly flattened, generally larger than macrophages, and had large lobular nuclei with marginated heterochromatin and prominent nucleoli (Figure 1a). Rough and smooth endoplasmic reticulum were plentiful, as were mitochondria, although the latter
Figure 1. a) Epithelioid cells in adherent monolayer prepared from peritoneal exudate cells. Pinosome-like vesicles are present along inner face of apposing cell membranes (arrowheads). A centriole (arrow) is present. (Bar = 1 μm). b) Degrading mitochondria (arrows) within epithelioid cell. (Bar = 0.5 μm). c) Desmosome joining adjacent epithelioid cells. Attachment plaques (arrowheads) and an electron-opaque intermediate line are present. (Bar = 0.1 μm).
frequently appeared swollen with degenerate cristae (Figure 1b). Cells were typically tightly apposed, and numerous small electron-lucent vacuoles resembling pinosomes were present in a single layer immediately beneath adjacent cell membranes. Desmosomes were frequently found between adjacent cells (Figure 1c). Individual desmosomes ranged from 0.1-0.2 μm in length and had a distinct electron-opaque intermediate line. Mycobacteria- and debris-containing phagosomes as well as labeled lysosomes were infrequently observed in this cell type. Centrioles were also occasionally observed.

**Infected cells**

Overall morphology of macrophages and phagosomes was similar at all time points (2-72 hours). Intracellular mycobacteria were always contained within membrane-bound phagosomes, and escape to the cytoplasm was not observed. Numbers of intraphagosomal mycobacteria ranged from one to greater than 40. Phagosomal morphology varied from conformant, in which the phagosomal membrane was tightly apposed to the mycobacterial cell wall, to expanded, in which space was present between bacilli and the phagosomal membrane. Percentage of conformant vs. expanded phagosomes did not differ significantly between live and HK mycobacteria treatments at 2 or 24 hours, or within treatments between the two timepoints (Student’s t-test, p>0.05). Expanded phagosomes typically contained larger numbers of mycobacteria than conformant phagosomes. The cell walls of both live and HK bacteria appeared largely intact, however HK bacteria were typically much less electron opaque and exhibited
coagulation and separation of the cytoplasm from the cell membrane. Large numbers of intact HK and live mycobacteria were present within macrophages for the duration of the study (72 hours). Obviously disrupted or degenerate bacilli were rare at all time points, although phagosomes containing debris and myelin figures were common.

Formalin-killed *Y. ruckeri* were typically found singly in phagosomes, and considerably lower numbers of macrophages contained intact FK *Y. ruckeri* than live or HK mycobacteria. Very few intact FK *Y. ruckeri* were found within macrophages at the 24 hour time point. Phagosomes containing unidentified debris were common in cells treated with FK *Y. ruckeri* at both 2 and 24 hours.

Macrophages containing HK or live *M. marinum* experienced some mortality, which was characterized by disintegration of organelles, cytoplasmic coagulation, and loss of membrane integrity. Intact bacilli were observed within cells even in advanced states of degradation. Dead or dying cells were phagocytosed by adjacent infected and uninfected macrophages, resulting in large phagosomes containing intact mycobacteria and/or cellular debris (Figure 2). In such cases, it was difficult to determine whether peroxidase reactivity or Au-BA within phagosomes resulted from passive accumulation of label via phagocytosis or by further fusions within the phagocytosing cell. As the “history” of each phagosome examined could not be determined, debris-containing phagosomes with peroxidase activity or gold beads were counted as fused.
Figure 2. Degrading cell with intact *M. marinum* (arrows) within phagosome of adherent macrophage. (Bar = 1 μm).
Labeled vesicles

Au-BA and peroxidase reactivity were found in membrane-bound vesicles that were observed to fuse with phagosomes. These will collectively be referred to as lysosomes, however caveats to this classification will be discussed.

Peroxidase-positive lysosomes were observed in some control cells that were not labeled with exogenous HRP. Endogenous peroxidase-positive vesicles frequently contained Au-BA, indicating their lysosomal status. Positive peroxidase reaction was also observed in *M. marinum*-containing phagosomes in some cells that did not receive exogenous HRP. Administration of exogenous HRP greatly increased both the number and staining intensity of intracellular peroxidase-positive compartments. Peroxidase-positive lysosomes were found in both the peripheral and perinuclear cytoplasm, and were present in >95% of macrophages receiving exogenous HRP at all time points. Circular and tubular profiles of peroxidase-positive lysosomes were observed. Internal structure generally could not be discerned due to the presence of the DAB reaction product, however the pattern of DAB label varied from solid black to a patchy, often margined, distribution on an electron-lucent background. (Figure 3a). Peroxidase-positive lysosomes in cells receiving exogenous HRP frequently contained Au-BA. In infected cells, peroxidase and/or gold-labeled lysosomes were usually found near phagosomes. In uninfected cells, labeled lysosomes were frequently found clustered together, often with close membrane apposition.

Gold-labeled lysosomes were polymorphic and ranged in size from approximately 0.1 to 1.0 μm. Au-BA-labeled lysosomes were observed in between 40 and 65% of the
Figure 3. a) Peroxidase-positive lysosomes (black) within adherent striped bass macrophage. Circular and tubular profiles are present, as well as profiles with marginated DAB reaction product (arrow). (Bar = 0.5 μm). b) Au-BA-labeled lysosomes. Gold beads (black dots) can be seen within membrane-bound vesicles. (Bar = 0.5 μm).
adherent cell population at 2 hours, depending on the individual fish. Contents of gold-labeled lysosomes were typically amorphous, but small round inclusions were occasionally present (Figure 3b). Density of Au-BA label ranged from one to greater than 100 beads.

**Ultrastructure of phagolysosomal fusion**

Phagosomes containing both live and HK *M. marinum* were fused with peroxidase- and Au-BA-positive lysosomes, as evidenced by the presence of DAB reaction product and gold beads within phagosomes, as well as by direct observation of fusion events (Figures 4a,b). Lysosomes appeared to integrate into the phagosomal membrane upon fusion, thus adding their membrane to that of the phagosome. Gold beads and DAB reaction product were separated from the bacterial plasma membrane by an electron-lucent cell wall (Figure 4c). The boundary between the cell wall and DAB reaction product, in particular, was sharply delineated, with a slight increase in electron-opacity at the interface. Cell walls were typically between 70-80 Å thick, and walls of adjacent bacteria frequently appeared confluent. In unfused phagosomes, the outermost margin of the cell wall appeared as a moderately electron-opaque line which was often discontinuous. Electron-transparent zones distal to the cell wall as described by other researchers (Frehel et al., 1986c; Rulong et al., 1991) were not observed.

The appearance of DAB reaction product within phagolysosomes was variable. Reaction product in conformant phagolysosomes typically filled all available space.
Figure 4. a) Live *M. marinum* in peroxidase- and gold-positive phagolysosome. Markers are present between bacilli (asterisk) and phagosomal membrane (arrowheads). (Bar = 0.5 μm). b) HK *M. marinum* in phagolysosome. Bacterial cytoplasm is coagulated and relatively electron-lucent, but an intact cell wall (arrows) is apparent. Fusion with a peroxidase-positive lysosome can be seen (arrowhead) (Bar = 1 μm). c) Detail of live *M. marinum* cell wall. Bacterial plasma membrane (bilayered, indicated by arrowhead) is separated from dark DAB reaction product by an electron lucent cell wall. The border between the cell wall and DAB reaction product is thickened and dense (open arrowhead). (Bar = 0.1 μm). d) Patchy DAB reaction product in phagosome containing live *M. marinum* (Bar = 0.5 μm). e,f) Phagolysosomes containing HK (e) and live (f) *M. marinum*. DAB reaction product is weak and patchy within phagolysosome, although multiple fusing lysosomes can be seen around phagolysosomal periphery (arrows). (Bars = 0.5 μm (e) and 0.1 μm (f)).

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between bacilli and phagosomal membranes. In some phagolysosomes, however, coagulation of the reaction product was observed (Figure 4d). In large, expanded phagolysosomes, DAB signal was often weak and patchy, although fusion events with peroxidase-positive lysosomes could be directly observed (Figures 4e,f).

Phagosomes containing FK Y. ruckeri were almost always conformant, and fusion with both peroxidase- and Au-BA-positive lysosomes was observed (Figure 5). FK Y. ruckeri within phagolysosomes often had disrupted morphology.

**Quantitation of phagolysosomal fusion**

Peroxidase reactivity was found in a high proportion (>70%) of phagosomes containing mycobacteria at all time points. Moderate (30-45%) rates of fusion with gold-labeled lysosomes were observed between 2 and 48 hours, with a relatively low fusion rate of 20% seen for the one fish sampled at 72 hours (Table 1). When phagosomes only from cells containing Au-BA were examined, however, gold beads were present in phagosomes at roughly comparable rates to peroxidase activity. At the two time points with sufficient numbers of fish for statistical comparison (2 and 24 hours), no differences were observed between phagosomes containing live and HK mycobacteria with respect to the presence of Au-BA or peroxidase activity. No significant differences were noted for either marker or treatment between 2 and 24 hours. (Student’s t-test, p>0.05).

Peroxidase reactivity and gold beads were found in phagosomes containing FK Y. ruckeri at considerably lower rates than in phagosomes containing mycobacteria. After a chase of 2 hours, these markers were present in 42.5 ± 7.7% and 15.3 ± 6.2% of FK Y.
Figure 5. Formalin-killed *Yersinia ruckeri* (asterisk) within adherent striped bass macrophage. Fusion with an peroxidase- and gold-positive lysosome is visible at lower right. (Bar = 0.5 μm).
Table 1. Percentage of live- or HK- *M. marinum* phagosomes (% ± SD) containing peroxidase activity (PO) or gold beads (Au-BA). Au-BA corr(ected) column indicates Au-BA fusion rates in only cells containing Au-BA.
<table>
<thead>
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<th>Live M. maritimus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Au-BA (corr.)</td>
<td>PO</td>
</tr>
<tr>
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<td>71.3±4.6</td>
<td>72.0±10.4</td>
</tr>
<tr>
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ruckeri phagosomes, respectively (mean ± SD, n=4). Because very few identifiable FK Y. ruckeri were present within macrophages at 24 hours, fusion rates were not assessed.

**Bactericidal assay**

Twenty-four and 48 hour outgrowth curves of known numbers of mycobacteria were superimposable between $2.1 \times 10^7$ and $6.6 \times 10^5$ cells well$^{-1}$ ($OD_{590} \sim 1.3 - 0.25$), and displayed a curvilinear relationship between MTT reduction and log # of bacteria per well (Figure 6a). Below $6.6 \times 10^5$ cells/well, $OD_{590}$ decreased by only small increments with two-fold dilutions of bacteria, especially in the 48 hour outgrowth curve. Seventy-two and 96 hour outgrowth curves displayed bimodality, with a secondary peak in MTT reduction at approximately 1000 original cells well$^{-1}$.

In the *in vitro* bactericidal assay, an overall reduction in numbers of *M. marinum* was observed between 0 and 48 hours, followed by a slight but nonsignificant increase at 72 hours (Figure 6b). Mean $OD_{590}$ at all time points was significantly different from $T_0$ by Dunnett’s test ($\alpha=0.05$). $KL_{AQ}$ was slightly but nonsignificantly lower than $KL_{OB}$ at all time points.
Figure 6a. Standard curve of MTT reduction (OD$_{590}$) vs. serial two-fold dilutions of a known quantity of *M. marinum*. Bacteria were incubated for between 24 and 96 hours at 30°C in 96-well plates containing 150 µl MDB and 50 µl lysis buffer well$^{-1}$. Ten µl MTT was added per well and color was developed for 4 hours at 30°C. Bacteria were lysed with 25 µl/well$^{-1}$ 10% SDS, formazan was solubilized overnight on a mechanical rocker, and plates were read at 590 nm on an automated plate reader. Points represent mean absorbance ± SD of quadruplicate wells. Actual bacterial numbers were estimated by transforming raw OD$_{590}$ values by the 48 hour curve. The curve was fitted with least-squares methods ($r^2=0.98$) to data points between 7.32 and 5.82 log bacteria well$^{-1}$, all of which had SD <10% of the mean.

Figure 6b. Persistence of *M. marinum* in adherent striped bass macrophages. Horizontal axis is time elapsed after removal of nonadherent extracellular bacteria and addition of fresh medium. Killing Index (KI) was calculated by dividing the mean values of quadruplicate wells at time $x$ ($T_x$) by mean values of quadruplicate wells at time 0 ($T_0$). KI$_{OD}$ is KI based on raw OD$_{590}$ values. KI$_{A_c}$ is KI based on raw OD$_{590}$ values transformed according to a standard curve of known mycobacterial numbers vs. OD$_{590}$. Columns indicate mean KI ± SD of 5 fish. Asterisks indicate significant difference of mean $T_x$ from mean $T_0$ (Dunnett’s test $\alpha=0.05$).
DISCUSSION

Exogenous horseradish peroxidase (HRP) has been commonly used as a fluid-phase marker for examining phagosome-lysosome fusion within mycobacteria-infected mammalian cells in vitro. Studies typically do not include controls for endogenous peroxidase as mammalian macrophages are generally considered to be peroxidase-negative (Frehel et al., 1986a; Marsh et al., 1986; de Chastellier et al., 1995; de Chastellier & Thilo, 1997). Fish monocytes and macrophages are also thought to lack peroxidase, in contrast to neutrophilic granulocytes, which possess strongly peroxidase-positive granules (Ellis, 1977). Peroxidase-positive vesicles have been demonstrated in rainbow trout (Oncorhynchus mykiss) macrophages, however, and it has been postulated that they are derived from neutrophils (Afonso et al., 1998a; Afonso et al., 1998b). Similar transfer of neutrophil granules to macrophages has also been documented in rodents (Sanui et al., 1982; Leung & Goren, 1989) and man (Parmley et al., 1981). In this study, addition of exogenous HRP markedly increased the number and staining intensity of peroxidase-positive vesicles within macrophages, however, controls for endogenous peroxidase revealed smaller numbers of peroxidase-positive vesicles within adherent macrophages, as well as endogenous peroxidase reactivity within phagosomes containing mycobacteria or cellular debris. The fine structure and location of endogenous peroxidase-positive vesicles were consistent with peroxidase-positive vesicles in cells given exogenous HRP. Additionally, gold beads were frequently found in peroxidase-
positive vesicles of both HRP-treated and control cells. These observations are consistent with the presence of lysosomes with endogenous peroxidase activity within striped bass macrophages. Administration of exogenous HRP therefore appeared to augment peroxidase reactivity in endogenous peroxidase-positive lysosomes in addition to labeling lysosomes previously negative for endogenous peroxidase. The possibility that incorporated peroxidase-positive material from neutrophilic granulocytes could have been responsible for peroxidase reactivity within phagosomes containing mycobacteria, however, somewhat complicates interpretation. On one hand, if neutrophil-derived material was incorporated into lysosomes, as is suggested by colocalization of gold beads, the peroxidase reactivity observed would be a true indicator of lysosomal fusion. Alternately, if neutrophil-derived material was delivered directly to phagosomes without passing through a lysosomal compartment, or if a portion of endogenous peroxidase-positive vesicles were nonlysosomal, lysosomal fusion rates based on peroxidase activity would have been overestimated.

Attenuation and patchiness of peroxidase activity was observed, especially in expanded phagosomes. In some cases, little or no DAB reaction product could be seen within the phagosome, despite direct observation of fusing lysosomes (Figures 4d,e,f). The pattern of DAB reaction product within lysosomes was also heterogeneous, with staining ranging from solid black to a marginated, patchy distribution (Figure 3a). Similar observations have been made by other researchers (Frehel et al., 1986a), and it has been suggested that vesicles with marginated reaction product represent early endosomes, and those with solid reaction product represent lysosomes (Frehel et al.,
1986a; de Chastellier & Thilo, 1997). In the present study, phagosomes with weak or patchy DAB reaction product were fused by solid black lysosomes, which often contained gold beads (Figure 4f). These observations indicate that the patchy HRP reactivity observed within phagosomes was not due to fusion with early endosomes. Further, as this staining pattern was observed in phagosomes containing both live and HK *M. marinum*, it is not likely that the attenuation of staining was due to the mycobacteria. Rather, it appeared that attenuation of the HRP signal was due to alteration or inactivation of the enzyme by conditions within the phagosome.

Because Au-BA has no endogenous occurrence in macrophages and is not susceptible to intraphagosomal attenuation, it was the more unequivocal marker of lysosomes in this study. The major disadvantage to this label was the observed heterogeneity in uptake by adherent macrophages. Whereas >95% of macrophages receiving exogenous HRP were peroxidase-positive, only between 40 and 65% of monolayer cells formed Au-BA-labeled lysosomes, depending on the individual fish. When only cells containing Au-BA marker were examined for Au-BA in *M. marinum* phagosomes, however, presence of gold beads was comparable to peroxidase activity. Although adherent macrophages were morphologically homogeneous, the fact that they were derived from primary culture makes it plausible that cells in a variety of activation states were present. Rates of endocytic uptake and lysosomal formation may thus have differed between cells, contributing to lack of Au-BA-labeled lysosomes in some. Stimulation of macrophages or the use of a monocyte-derived cell line may improve future studies examining lysosomal fusion using Au-BA as a lysosomal marker, although
these strategies may introduce additional variables that may not mirror the situation in vivo.

Despite the marker difficulties described above, this study found evidence that the fish pathogen *Mycobacterium marinum* occupies a fusiogenic phagosome in macrophages of striped bass in vitro. Despite contact with lysosomal contents, *M. marinum* remained intact and viable for up to 72 hours as evidenced by ultrastructural observation (Figure 4) and bactericidal assay (Figure 6). These findings are seemingly incongruous with a large body of literature indicating that pathogenic mycobacteria of mammals occupy unfused phagosomes that do not mature (Brown et al., 1969; Armstrong & Hart, 1971; Crowle et al., 1991; Sturgill-Koszycki et al., 1994; Clemens & Horwitz, 1995; Russell, 2001). Observation of pathogenic mycobacteria occupying fusiogenic vacuoles, however, is not without precedent. The mouse pathogen *M. lepraemurium* has been shown to persist in fused phagolysosomes (Hart et al., 1972; Hart et al., 1987), and lysosomal fusion with phagosomes containing live *M. tuberculosis* H37Rv has been induced by pre-incubation of bacteria with rabbit serum (Armstrong & Hart, 1975). High rates of lysosomal fusion with phagosomes containing live and HK *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. bovis* BCG, and *M. smegmatis* have been observed during the first two days of infection in mouse macrophages (McDonough et al., 1993). Fusion rates then declined at 3 days for phagosomes containing both strains of live *M. tuberculosis*, a phenomenon the authors attributed to budding of bacilli into nonfused phagosomes or escape into the cytoplasm. *M. marinum* phagosomes observed in the present study did not appear to subdivide, and phagosomal membranes were always observed around intracellular
bacteria. Also, fusiogenicity of *M. marinum* phagosomes did not appear to decrease over time, although the study was only extended to 72 hours, and sample size did not allow statistically valid comparisons between 2/24 hours and later time points.

The intracellular behavior of *M. marinum* has been examined in mammalian cells. In mouse RAW 264.7 cells, compartments containing live *M. marinum* did not acidify and excluded vacuolar ATP-ase relative to those containing UV- or heat-killed bacteria, respectively (Barker et al., 1997). Additionally, heat-killed *M. marinum* colocalized with label for the late endosomal/lysosomal markers M6PR and cathepsin D at higher levels than live *M. marinum*. Therefore, *M. marinum* appeared to behave similarly in mouse cells to mycobacteria such as *M. tuberculosis* and *M. avium*. In contrast, Mor et. al. (1985) demonstrated high (>90%) rates of fusion between *M. marinum* phagosomes and ferritin-labeled lysosomes within primary cultures of mouse peritoneal cells in vitro.

Using acidotropic fluorescent dye in mouse bone marrow-derived macrophages, Via et. al. (1998) demonstrated the rate of *M. marinum* colocalization with acidic compartments to be intermediate to *M. bovis* BCG and *M. smegmatis*.

The present study and a previous study of *M. marinum* in fish macrophages appear to be similarly inconsistent. Whereas we found high rates of lysosomal fusion with phagosomes containing live and HK *M. marinum* in striped bass macrophages, others have demonstrated inhibited fusion of thorium dioxide-labeled lysosomes with *M. marinum* phagosomes as compared to those of the nonpathogenic *M. smegmatis* (El-Etr et al., 2001). Gold beads and peroxidase activity were found within *M. marinum* phagosomes in the present study at rates similar to that of thorium dioxide in *M.
*smegmatis* phagosomes observed previously (El-Etr et al., 2001). As these studies were conducted with different cell sources (striped bass primary culture vs. carp monocytic cell line) at different temperatures (12°C vs. 28°C), it is possible that intracellular behavior of *M. marinum* may vary with host cell type and experimental conditions. Additionally, activation state of macrophages may influence phagolysosomal fusion, as evidenced by studies showing increased fusion of late endosomal/lysosomal compartments with phagosomes containing mycobacteria in macrophages stimulated with IFN-γ or lipopolysaccharide (LPS) (Sibley et al., 1987; Schaible et al., 1998; Via et al., 1998).

Although HK *M. marinum* were shown to be nonviable via plating on MDA and possessed obviously disrupted cytoplasm, the integrity of their cell walls was largely uncompromised, and recognizable intracellular HK bacilli were present for the duration of the study. Resistance of killed mycobacteria to intracellular digestion has been previously noted (Armstrong & Hart, 1975; Frehel et al., 1986a). As no protective factors could have been actively secreted by HK *M. marinum*, their persistence in phagolysosomes must be attributed to properties of the cell wall. The mycobacterial cell wall is composed largely of long-chain mycolic acids which form an impermeable hydrophobic layer that confers resistance to environmental conditions, disinfectants, and antibiotics (Jarlier & Nikaido, 1990; Russell, 1999). Ultrastructural observations in this study showed the cell wall of both live and HK *M. marinum* effectively separated the mycobacterial plasma membrane from DAB reaction product and gold beads. This is suggestive of an overall cell wall impermeability, although the possibility that some lysosomal contents were able to penetrate the cell wall cannot be excluded.
Phagosomes are thought to undergo a dynamic process of maturation prior to formation of the phagolysosome. In this process, termed “kiss-and-run” by Desjardins, the phagosome is remodeled via sequential interaction with biochemically distinct populations of endosomes. (Desjardins et al., 1994b; Desjardins, 1995; Duclos et al., 2000). Recent studies have demonstrated that pathogenic mycobacteria have the capability to modulate the normal maturation of the phagosome, retaining fusogenicity with early endosomes while inhibiting fusion with late endosomes and lysosomes (Sturgill-Koszycki et al., 1994; Xu et al., 1994; Clemens & Horwitz, 1995; Clemens, 1996; Russell et al., 1996; Clemens et al., 2000a; Clemens et al., 2000b). This strategy may allow mycobacteria to retain access to nutrients and growth factors while avoiding degradation.

While a number of markers for early/late endosomes and lysosomes have been developed for mammalian cells, the biochemical composition of fish endosomes and lysosomes has not yet been characterized, so definitive categorization of endosome/lysosome-like bodies in fish cells is not currently possible. Therefore, the possibility must be considered that a portion of the labeled vesicles that were observed fusing with phagosomes in this study represent early endosomes rather than mature primary lysosomes. Particularly with respect to Au-BA, this would be inconsistent with a large volume of mammalian literature, however, it is one possible explanation for why mycobacteria contained in phagolysosomes were not killed or digested. Biochemical characterization of the endosomal/lysosomal network in fish macrophages will be

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necessary to further elucidate the position of this bacterium in the endosomal/lysosomal network of striped bass cells.

A morphologically distinct cell population was observed among adherent macrophage monolayers in this study. These cells were ultrastructurally highly similar to epithelioid cells observed previously in *M. marinum* granulomas of striped bass (Gauthier et al., 2004). They possessed increased nuclear and cytoplasmic volume, large quantities of endoplasmic reticulum, and in many cases were joined by desmosomes. Desmosomes, normally considered to be a characteristic of true epithelium in mammals, have been described previously in epithelioid cells of fish granulomas (Noga et al., 1989; Gauthier et al., 2004). Due to the ultrastructural similarities between these cells and epithelioid cells of *M. marinum* granulomas produced *in vivo*, it is likely that these cells are in fact epithelioid macrophages. Epithelioid transformation *in vitro* would not be unexpected, as it has been observed previously for avian macrophages (Sutton & Weiss, 1966). It is also possible that the epithelioid cells observed in this study had begun or completed transformation within the peritoneal cavity prior to lavage and adherence. Epithelioid cells were observed in control (uninfected) preparations as well as those receiving live and HK *M. marinum*, so it does not appear that their formation was due to the presence of mycobacteria. Epithelioid cells possessed few peroxidase- or Au-BA-labeled vesicles and very rarely contained mycobacteria, so they were not included in the analysis of phagolysosomal fusion. The significance of degenerating mitochondria in some epithelioid cells is unclear, but it is possible that *in vitro* conditions were not appropriate to their maintenance.
Relative to live and HK *M. marinum*, peritoneal macrophages of striped bass appeared to be poorly phagocytic for FK *Yersinia ruckeri*. Additionally, peroxidase activity and gold beads were found in FK *Y. ruckeri* phagosomes at considerably lower rates than with phagosomes containing live or HK *M. marinum*. The fact that very few intact FK *Y. ruckeri* could be observed within cells at 24 hours suggests that they were degraded rapidly by adherent macrophages. Therefore, it is plausible that FK *Y. ruckeri* in fused phagolysosomes were not identified due to degradation, and only bacilli in phagosomes that had not yet fused were counted. Alternately, FK *Y. ruckeri* phagosomes may not elicit lysosomal fusion at high rates, but instead bacteria may be degraded by other means such as phagosomal acidification or respiratory burst. FK *Y. ruckeri* were chosen for this study because of ready availability and known capacity for being phagocytosed by fish macrophages. The low occurrence of FK *Y. ruckeri* within striped bass macrophages and apparently low rate of lysosomal fusion with phagosomes containing FK *Y. ruckeri*, however, indicates that this particle was not optimal for comparison to mycobacteria. Future studies should examine other degradable and/or nondegradable particles for control purposes, with optimal characteristics being high rates of internalization and lysosomal fusion.

Bactericidal activity was determined in this study using the reduction of MTT as a proxy measure for bacterial numbers. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a soluble yellow tetrazolium dye that is reduced to purple formazan product by the action of bacterial dehydrogenases in proportion to the number of bacteria present (Peck, 1985). The MTT bactericidal assay has been employed in
several instances to measure bactericidal activity of fish phagocytes against various bacterial pathogens (Graham (nee Chung) et al., 1988; Skarmeta et al., 1995; Roszell & Anderson, 1996) and has been used to measure rifampin resistance of *M. tuberculosis* and *M. bovis* BCG (Mshana et al., 1998). This assay is considerably less time- and labor-intensive than the traditional method of direct colony counting. Additionally, colorimetric measurement of mycobacteria circumvents potential enumeration error due to “clumping,” or the strong tendency of mycobacteria to self-aggregate in broth culture and within cells.

A period of time between cell lysis and MTT addition is typically allowed for outgrowth of bacteria to levels that can reduce MTT in sufficient quantity to be spectrophotometrically detected. As demonstrated in Figure 6a, the time of outgrowth for *M. marinum* was critical to the analysis of data. Whereas the reduction of MTT decreased with decreasing bacterial numbers after 24 and 48 hours of outgrowth, the 72 and 96 hour curves were bimodal, with a secondary peak around 1000 original bacterial cells. The reasons for these secondary peaks are unknown, but their presence indicates that *M. marinum* should not be outgrown for longer than 48 hours before addition of MTT. Additionally, the 24 and 48 hour curves demonstrated that the MTT assay has a fairly narrow working range for the enumeration of *M. marinum*. For the raw OD$_{590}$ values observed in this study (range 0.582-0.386), either 24 hour or 48 hour outgrowth periods were appropriate, as these points fall within the portion of the standard curve in which MTT reduction strongly decreases with decreasing bacterial numbers. For lower bacterial concentrations (~$10^6-10^5$), however, a 24 hour outgrowth period would have
been more appropriate, as the rate of MTT reduction decreased by only a small amount over this range after 48 hours of outgrowth. The 24 hour curve, however, reached background levels at ~10^4 bacteria well^-1, indicating a lower detection limit for the assay. A similarly narrow working range for the MTT assay has been noted for *M. bovis* BCG by Mshana et al. (1998), with relatively high bacterial densities (2 x 10^7 – 1 x 10^9) being necessary to fall within the linear portion of standard curves. These findings indicate that caution must be exercised when applying the MTT assay to enumeration of mycobacteria in experimental systems where bacteria will be initially introduced in or reduced to low numbers. In these instances, traditional plate-counting assays would be more appropriate, despite logistical difficulty and issues with clumping.

Studies using MTT methodology frequently report results as Killing Index, which is mean OD at time x divided by mean OD at time 0. This measure is often converted directly to percent bacterial survival by multiplying KI by 100. This direct conversion from KI to percent bacterial survival is only accurate, however, when the relationship between MTT reduction and bacterial numbers is linear with a slope of 1, i.e.: when a two-fold decrease in bacterial numbers will result in a two-fold decrease in OD. If the slope of this line is <1, OD will decrease at a lesser relative rate than bacterial numbers, and consequently percent killing will be underestimated while percent outgrowth will be overestimated. The inverse applies for OD/bacterial number relationships with slopes greater than 1. For this reason, Killing Index was calculated as both KI_{50} and KI_{Act}. As the slope of the standard curve representing the OD_{590} values observed in the bactericidal assay was near 1, both measures are similar.
Quantitative bactericidal assay revealed a significant decrease in bacterial numbers at 24-72 hours relative to time 0, with mean KIАт values reaching as low as 65.8% at 48 hours. Despite this apparent bactericidal activity, however, most of the initial infective dose remained viable throughout the course of the assay. Several hypotheses may be presented to explain this apparent heterogeneity in bacterial susceptibility. First, although fluorescent staining revealed very high viability of *M. marinum* after processing for single cell suspension, it is possible that a portion of the bacteria may have been damaged to the point that they became susceptible to intracellular killing, but not to the point that the propidium iodide dye could penetrate the cell and mark the bacterium as nonviable. Alternately, as primary cultures of peritoneal exudate cells were used in these assays, heterogeneity in macrophage activation state and bactericidal competence are to be expected. Therefore, if a subpopulation of cells in the monolayer was phagocytic but not inherently capable of generating an appropriate response to kill *M. marinum*, bacteria in those cells would be “protected” from degradation in more competent cells. Very few intracellular degrading bacilli were observed ultrastructurally, although debris-containing phagolysosomes were observed in most cells after treatment with mycobacteria. As few transitional stages between intact mycobacteria and unidentified debris were observed, it appears that degradation of intracellular *M. marinum*, when it occurred, was a rapid event.

Although the 12°C temperature used in these assays is not optimal for growth of *M. marinum*, transverse division of bacilli was occasionally observed ultrastructurally, indicating that replication did occur. This was reflected in the slight, but nonsignificant,
increase in mean KI that was observed at the 72 hour timepoint. Wild striped bass are found in a wide variety of water temperatures, ranging from less than 10°C in winter months to greater than 24°C during summer months in Chesapeake Bay. Therefore, the 12°C temperature used in these assays is relevant to striped bass for a portion of the year. Low temperatures have been shown to be suppressive to T lymphocyte responses and T-dependent B lymphocyte responses in cold- and warm-water fish species (Avtalion, 1969; Clem et al., 1984; Miller & Clem, 1984; Stolen et al., 1984; Bly & Clem, 1992). Phagocytosis and respiratory burst activity of fish phagocytes appear to be less sensitive to low temperatures, leading several researchers to postulate that non-specific immune responses are relatively important to overall protection at low temperatures. (Ainsworth et al., 1991; Collazos et al., 1994; Le Morvan et al., 1997). Further studies will be necessary to examine the relative rates of phagolysosomal fusion in striped bass macrophages infected with \textit{M. marinum} at various temperatures.

\textit{M. marinum} is phylogenetically similar to \textit{M. tuberculosis} (Rogall et al., 1990; Tonjum et al., 1998) and produces chronic granulomatous disease in fish (Talaat et al., 1998; Gauthier et al., 2003) and frogs (Ramakrishnan et al., 1997) that is similar in many respects to human tuberculosis. \textit{M. marinum} grows considerably faster in culture than \textit{M. tuberculosis}, and although it can cause granulomatous lesions of the extremities in humans, it is considered a biosafety level 2 organism, rather than the BSL 3 of \textit{M. tuberculosis}. Consequently, there has been considerable interest in the use of \textit{M. marinum} as a model organism for the study of mycobacterial pathogenesis in mammals (Ramakrishnan & Falkow, 1994; Ramakrishnan et al., 1997; Barker et al., 1998; Davis et
al., 2002; Solomon et al., 2003). The observed high rate of lysosomal fusion with *M. marinum* phagosomes in striped bass macrophages of this study, however, differs markedly from the nonfusion pattern observed by other researchers. Further study will be necessary to determine the extent to which the intracellular behavior of *M. marinum* differs between host cells, and whether the nonfusion pattern can be induced in striped bass macrophages by varying experimental conditions. Such work clearly has the capacity to further elucidate the intracellular interaction between mycobacteria and poikilothermic macrophages, and to strengthen the *M. marinum* model for mycobacterial pathogenesis.
GENERAL CONCLUSIONS

Chapter 1

1) *Mycobacterium marinum* given in a sublethal dose (~$10^5$ colony-forming units fish$^{-1}$) intraperitoneally to striped bass (*Morone saxatilis*) produced chronic granulomatous disease primarily of the mesenteries, spleen, and anterior kidney.

2) Inflammatory foci produced in the early stages of *M. marinum* infection developed into epithelioid granulomas, which in turn developed necrotic centers surrounded by several layers of highly compressed epithelioid cells (spindle cells). Acid-fast bacilli (AFB) were rare to absent by standard Ziehl-Neelsen (ZN) staining during the early (<6 week) development of granulomas.

3) Beginning at 8 weeks post-injection (p.i.), AFB became visible in large numbers within well-developed *M. marinum* granulomas. AFB were concentrated in spindle cell and core regions of lesions.

4) In mesenteries, development of bacillary granulomas was accompanied by healing of tissue and restoration of normal tissue architecture external to lesions. Large numbers of eosinophilic granule cells present within resolving inflammatory tissue suggested a role for this cell in the healing process. Pathology in spleen and anterior kidney was limited to granulomas.

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5) Beginning at 26 weeks and progressing to 45 weeks p.i., a secondary phase of recrudescent disease was observed in some fish. Recrudescence was characterized histologically by granuloma disintegration, reappearance of inflammatory leukocytes, fibrosis, and in some cases, partial or total destruction of affected organs. AFB were observed scattered throughout inflammatory tissue.

6) *M. marinum* quickly (≤2 weeks) established densities of >10⁴ CFU g⁻¹ in splenic tissue. Splenic densities increased over the course of the study, reaching in excess of 10⁹ CFU g⁻¹. Splenic bacterial density and disease stage were positively correlated.

7) Evidence was found for chromophobic forms of *M. marinum* in experimentally infected striped bass. Pre-oxidation of tissue sections with 10% periodic acid (P₂O₅) revealed acid-fast bacilli in lesions previously negative by ZN staining. This technique, however, increased background staining levels of tissue to the point where it would not likely be practical for routine diagnostics.

8) Preliminary evidence for horizontal transmission of *M. marinum* via water or detritus was found by examining unexposed fish in a holding tank receiving effluent from tanks holding infected fish. One of 7 fish in this tank developed epithelioid granulomas in anterior kidney and liver, but had no pathology in mesenteries or spleen. *M. marinum* was recovered from the spleen of this fish at 3.7 x 10⁵ CFU g⁻¹.

9) Granulomatous skin lesions as seen in wild Chesapeake Bay striped bass were not observed in fish experimentally infected with *M. marinum*, even in fish with severe internal disease.
10) Both *M. shottsii* and *M. gordonae* established persistent infections in the spleen of injected striped bass. Minor granulomatous inflammation was observed in mesenteries, while no pathology was observed in spleen or, with one exception, anterior kidney. Bacillary granulomas were observed in the mesenteries of only one *M. shottsii*- and two *M. gordonae*-infected fish over the course of the study.

This study is the first to experimentally examine chronic mycobacteriosis in the striped bass. Acute mycobacteriosis has been produced previously in this fish with very high initial doses of *M. marinum* (Wolf & Smith, 1999). These experiments, however, were only extended to 8 days p.i., at which time significant mortality of infected bass occurred. Mycobacteriosis in Chesapeake Bay striped bass appears to be a chronic disease, therefore long-term study of bass initially inoculated with sublethal doses of mycobacteria likely better mirrors the pathobiology of the disease in the wild.

Chronic mycobacteriosis produced in this study was multiphasic, with an apparently successful host response followed by severe secondary disease. This is significant, as it indicates an apparently successful granulomatous response to mycobacteria in fish may fail over time, leading to a secondary disease state considerably more severe than the primary. The factor(s) that led to the recrudescence of *M. marinum* disease in experimentally infected striped bass are unknown. A water temperature shift from 18°C-21°C did occur after the 26 week sampling, suggesting temperature may have played a role, however, recrudescent lesions were present in one fish before the temperature shift, making this association unclear.
Granulomatous skin lesions were not observed in this study, even in fish with severe visceral disease. This observation is another indication that factors not present in the laboratory study affect the pathogenesis of disease in wild fish. A crucial question to be answered by future research is whether visceral and dermal mycobacteriosis are two manifestations of the same disease (i.e.: Can mycobacteria in the viscera be disseminated to the dermis/epidermis, or vice-versa?), or two separate disease processes, initiated individually, possibly by different etiologic agents. Additionally, an intermediate scenario may be postulated whereby one disease process (visceral or dermal) may influence progression of the other via immunomodulatory effects such as induction of delayed-type hypersensitivity or alteration of cytokine expression.

*M. shottsii* established persistent splenic infections of striped bass in this study, but did not produce pathology in the spleen. This is inconsistent with the severe splenic disease seen in wild Chesapeake Bay striped bass from which *M. shottsii* was the only isolate. Several hypotheses may be presented to explain this finding. First, *M. shottsii* may require time periods longer than the duration of this study (45 weeks) to produce disease. Second, additional factors that were not present in the experimental design such as elevated temperature, fish stress, or repeated exposure to mycobacteria may be necessary for expression of disease due to *M. shottsii*. Third, the virulence of *M. shottsii* may become quickly attenuated in culture, or multiple strains with variable pathogenicity may exist.

The establishment of persistent splenic infection by *M. gordonae* in this study was unexpected, as *M. gordonae* is typically considered to be a nonpathogenic saprophyte.
The significance of this finding to disease in wild fish is unknown, as *M. gordonae* was introduced artificially rather than by natural exposure. The capability of *M. gordonae* to enter and establish itself in the spleen of striped bass, however, suggests that, given an appropriate portal of entry, presumably nonpathogenic environmental mycobacteria have the potential to be involved in production of disease.

**Chapter 2**

1) *M. marinum* injected intraperitoneally were phagocytosed within 24 hours by cells morphologically consistent with macrophages. Phagocytosed bacilli were contained within phagosomes that appeared to fuse with lysosomes based on the presence of electron-opaque material within the phagosomal membrane. *M. marinum* within apparently fused phagolysosomes did not appear to degrade.

2) Macrophages containing phagocytosed *M. marinum* rapidly aggregated into large masses, with adjacent cells linked by filopodial interdigitations.

3) Epithelioid transformation of macrophages in developing *M. marinum* granulomas involved increases in cell and nuclear volume, as well as mitochondria and endoplasmic reticulum content. Adjacent epithelioid cells elaborated desmosomal junctions. Spindle cell layers surrounding necrotic lesion cores appeared to be formed by compression of overlying epithelioid layers.

4) As observed histologically, few mycobacteria were found within developing epithelioid granulomas or early spindle cells granulomas. Ultrastructural
appearance of large numbers of mycobacteria in spindle cell and core regions of granulomas correlated with histological observations.

5) Crystalloids, possibly derived from eosinophilic granule cells, were commonly observed within *M. marinum* granulomas.

6) Granuloma disintegration was characterized by loss of epithelioid cell compression, development of a ragged lesion margin, and reappearance of inflammatory cells, primarily macrophages, on the lesion periphery.

7) Recrudescence of lesions was characterized by a loose organization of macrophages, fibroblasts, eosinophilic granule cells, and necrotic cellular material. *M. marinum* was observed within macrophages.

This study represents the first ultrastructural description of mycobacterial granuloma development in fish over an extended time period. Ultrastructural findings of Chapter 2 largely support the histological findings from Chapter 1. The extremely large numbers of AFB observed in bacillary granulomas appeared to originate from a small number of initial bacilli surviving within core and/or spindle cell layers, rather than from large numbers of non-histologically detectable alternate forms. Several interesting ultrastructural features were noted, including Charcot-Leyden-like crystalloids and desmosomes between epithelioid cells of granulomas.

In addition to description of granuloma formation, the early response of peritoneal exudate cells to injected mycobacteria *in vivo* was examined. The results suggest that *M. marinum* is contained within fused phagolysosomes *in vivo*, in contrast to the widely held
view that pathogenic mycobacteria survive intracellularly by inhibiting phagolysosomal fusion.

Chapter 3

1) *M. marinum* phagosomes in adherent striped bass macrophages were fused with lysosomes *in vitro*.

2) *M. marinum* remained intact and viable within striped bass macrophages for up to 3 days, indicating that they were resistant to degradation by lysosomal enzymes.

3) Mortality of *M. marinum*-infected macrophages occurred *in vitro*, and dead cells were phagocytosed by adjacent live cells. *M. marinum* remained intact in these phagosomes, while cellular components were degraded.

4) No significant differences were found in the rate of lysosomal fusion with phagosomes containing live and heat-killed (HK) *M. marinum*.
The results of this study indicated that phagosomes containing live or HK *M. marinum* were frequently fused by lysosomes in striped bass macrophages *in vitro*. Both live and HK *M. marinum* were resistant to degradation within phagolysosomes. This conflicts with previous reports of the *in vitro* behavior of mycobacteria within fish phagocytes (El-Etr et al., 2001), and is contrary to the generally held view that pathogenic mycobacteria survive intracellularly by inhibiting phagolysosomal fusion. This and other studies (Armstrong & Hart, 1975; Mor, 1985; McDonough et al., 1993; Bouley et al., 2001) that have shown pathogenic mycobacteria to occupy a fusiogenic phagosome suggest that mycobacteria may utilize multiple intracellular survival strategies depending on host cell and experimental conditions. Further examination of the behavior of *M. marinum* within striped bass macrophages, including the effects of temperature and macrophage activation, may provide valuable information for the development of *M. marinum* infection of poikilotherms as a model system for mycobacterial pathogenesis.

**Ongoing Research**

Although the laboratory exposure studies described in Chapter 1 are extremely valuable in exploring the pathogenesis of various mycobacterial species in striped bass, they are by nature highly artificial, and may lack crucial factors that influence the expression of disease in wild fish. One potential factor we are currently exploring is the effect of water temperature on the progression of disease due to *M. marinum* and *M. shottsii*. In order to more fully understand mycobacteriosis in striped bass, future studies
will also be needed to determine the effects of salinity, fish stress, and mode of entry (e.g. oral or bath exposure) on the progression of disease.

In addition to laboratory research, continuing field studies on wild Chesapeake Bay striped bass will be necessary to track the severity of the mycobacteriosis epizootic, determine potential risk factors predisposing striped bass to disease, and provide fisheries managers with information about the impact of the disease on wild stocks. Since 2002, a collaborative effort between VIMS and the National Fish Health Research Laboratory (USGS) has been underway to survey mycobacteriosis in striped bass from the Nanticoke, Potomac, York, and Rappahannock tributaries of Chesapeake Bay. Fifty fish from each river are taken from pound nets in late October / early November and assessed grossly and histologically for dermal and visceral mycobacteriosis. In addition, splenic cultures are performed to determine the identity and relative densities of _Mycobacterium_ spp. present.

While pound nets are a convenient way to obtain large numbers of striped bass from tributaries, there are certain spatial and population biases inherent in this collection method. The majority of striped bass collected in pound nets during the fall are small, nonmigratory males, which tend to have higher splenic lesion prevalence rates than females, possibly due to their longer residence time in the Bay (Cardinal, 2001). Therefore disproportionate representation of males in pound net samples may lead to overestimation of disease prevalence in the overall population of fish entering Chesapeake Bay. Also, pound net sampling is extremely localized and may not be accurately representative of fish populations over a larger spatial scale.
In an effort to expand the monitoring of mycobacteriosis in striped bass to the mainstem of the Bay, I have initiated a collaborative effort with the Chesapeake Bay Multispecies Monitoring and Assessment Program (ChesMMAP), VIMS Fisheries Department. ChesMMAP is a baywide trawl survey that collects data on adult fish species in order to determine abundance indices by age and derive population age structure data. Gut content analysis is also performed to establish predator-prey relationships necessary for multi-species assessment models. In addition, environmental parameters such as temperature, salinity, and dissolved oxygen are measured. Sampling is by a stratified-random design with five cruises per year covering 90 stations from the Susquehanna Flats to the Bay mouth. Since March 2003, I have been histologically surveying striped bass from the ChesMMAP trawl for dermal and splenic mycobacteriosis. The resulting 2003/2004 dataset should provide valuable information about the spatial and temporal distribution of diseased fish in Chesapeake Bay, prevalence and severity of infection by age and sex, and relationship between disease severity and condition factor. This information has the potential to improve our understanding of when and where fish become diseased, how environmental conditions affect the progression of disease, and how the disease impacts the health of infected fish.

One of the major gaps in our understanding of mycobacteriosis in Chesapeake Bay striped bass is uncertainty as to which of the several *Mycobacterium* spp. isolated from striped bass are pathogenic. Culturing of mycobacteria from splenic tissue of striped bass is extremely time- and labor-intensive, and the necessary aseptic conditions for tissue collection are not always logistically feasible. Therefore, there is a need for
development of additional, convenient tools for detection and speciation of *Mycobacterium* spp. in striped bass tissue. Toward this goal, I have begun development of peptide nucleic acid (PNA) molecular probes to detect mycobacteria in formalin-fixed, paraffin-embedded tissue sections by *in situ* hybridization. *Mycobacterium* genus-specific and universal bacterial probes designed to recognize 16S (small subunit) rRNA are currently being optimized, with the latter serving as a positive control for the former. The high degree of conservation among *Mycobacterium* spp. of 16S rRNA sequence does not make this gene a promising candidate for development of species-specific probes. Therefore, 23S (large subunit) rRNA sequences are currently being obtained from *Mycobacterium* spp. isolated from striped bass in Chesapeake Bay. It is hoped that the larger size of this gene will provide more areas of sequence that are divergent between species, therefore making development of species-specific probes possible. These probes will allow detection of individual *Mycobacterium* spp. in the context of pathology produced in host tissues. This will enable simultaneous assessment of both infection and disease, a capability lacking in the currently available methods of histology, culture, and PCR. In addition, surveys of striped bass tissues archived since 1997 will be possible, allowing retrospective analysis of the development of the current epizootic.
APPENDIX

Chapters 1 and 2 of this work represent a collaborative effort by several researchers. M. Rhodes and H. Kator performed isolation and enumeration of mycobacteria from the spleen of injected striped bass, as well as statistical analysis of bacteriologic data. W. Vogelbein provided instruction on interpretation of histologic and ultrastructural data. Exposure studies were performed in the laboratory of C. Ottinger, who designed and maintained the aquaria, and performed fish husbandry for the duration of the study. All authors made significant contributions to the initial design of the exposure studies.
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