The Morphology, Development, and Function of Experimentally Induced Macrophage Aggregates in Rivulus Marmoratus.

Wolfgang Klaus Vogelbein

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The morphology, development, and function of experimentally induced macrophage aggregates in *Rivulus marmoratus*

Vogelbein, Wolfgang Klaus, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1991
THE MORPHOLOGY, DEVELOPMENT, AND FUNCTION OF EXPERIMENTALLY INDUCED MACROPHAGE AGGREGATES IN RIVULUS MARMORATUS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Interdepartmental Program in Veterinary Medical Sciences

by

Wolfgang K. Vogelbein
B.S., Southampton College, 1976
M.S., California State University, Long Beach, 1981
May 1991
ACKNOWLEDGMENTS

The completion of this dissertation would not have been possible without the support and help of several people. I extend my sincere gratitude to Drs. Robin M. Overstreet and Ronald L. Thune for their unwaiving confidence in my abilities and their support and guidance during each phase of my program of study. Special thanks are due to Dr. Overstreet for generously providing me with the financial support to attend several national and international scientific meetings. I gratefully acknowledge Dr. Thomas R. Klei for the use of his diamond knife and Drs. William G. Henk and William E. Hawkins for providing access to the Electron Microscopy Facilities at the LSU School of Veterinary Medicine and Gulf Coast Research Laboratory, respectively. I thank the members of my Graduate Committee for their help. Your friendship and the varied discussions we had, both during lucid moments as well as under the influence of boiled crawfish and beer, have meant a lot to me. I thank Dr. William P. Davis of the U.S. Environmental Protection Agency, Gulf Breeze, Florida, for supplying laboratory-reared Rivulus marmoratus. I gratefully acknowledge Dr. William W. Walker and Ms. Cherie S. Heard for assistance with the cadmium exposure studies. I thank Dr. Thomas F. Lytle for assistance with the analytical chemistry and Mr. John Ogle for the access to his image analysis system. I thank my good friend Jack W. Fournie for his generous help with many aspects of my research and life. Finally I thank my wife and best friend, Mary Ann for her help and support and for enduring without complaint the completion of this dissertation. This work was funded partially by a Fellowship from the Mississippi-Alabama Sea Grant Consortium, under grant NA85AA-D-SG005, the National Marine Fisheries Service under Pub. L. 88-309, Project No. 2-422-R, and the U.S. Department of Agriculture, Cooperative State Research Service, Grant No. 88-38808-3319.
TABLE OF CONTENTS

ACKNOWLEDGMENTS .................................................... ii
LIST OF FIGURES .................................................... vi
LIST OF TABLES .................................................. viii
ABSTRACT .......................................................... ix
CHAPTER 1: INTRODUCTION ......................................... 1
   EARLY STUDIES ............................................... 2
   MORPHOLOGY OF MACROPHAGE AGGREGATES ......................... 3
   PHYLOGENETIC AND ONTOGENETIC RELATIONSHIPS ................. 6
   MACROPHAGE AGGREGATE FUNCTIONS ............................. 7
      Tissue Catabolism ..................................... 7
      Immunological Studies ................................. 8
      Disease Associations ................................... 9
      Toxicologic Associations .............................. 12
   RATIONALE AND HYPOTHESES OF THE STUDY ..................... 14
   REFERENCES CITED ........................................... 18
CHAPTER 2: SEQUENTIAL DEVELOPMENT AND MORPHOLOGY OF
EXPERIMENTALLY INDUCED HEPATIC MACROPHAGE AGGREGATES IN RIVULUS
MARMORATUS ........................................................ 25
   PERMISSION TO RE-MICROFILM PUBLISHED MANUSCRIPT. ........ 25
   ABSTRACT .................................................... 26
   INTRODUCTION ................................................ 27
   MATERIALS AND METHODS ..................................... 28
   RESULTS ...................................................... 29
   DISCUSSION .................................................. 31
   ACKNOWLEDGMENTS ........................................... 33
   FIGURES ...................................................... 34
   REFERENCES .................................................. 40
CHAPTER 3: THE ULTRASTRUCTURAL AND HISTOCHEMICAL CHARACTERIZATION OF EXPERIMENTALLY INDUCED HEPATIC MACROPHAGE AGGREGATES IN *RIVULUS MARMORATUS* (PISCES: CYPRINODONTIDAE) .................................................. 42

**INTRODUCTION** .................................................. 42

**MATERIALS AND METHODS** ........................................ 43

**RESULTS** .................................................................. 45

  Hepatic Macrophage Aggregate Formation .......................... 45
   5-12 Days Post-Infection ........................................... 45
   15 Days Post-Infection .............................................. 46
   18 Days Post-Infection .............................................. 46
   20 Days Post-Infection .............................................. 47
   30-40 Days Post-Infection ........................................... 47
   50-80 Days Post-Infection ........................................... 48
   100-250 Days Post-Infection ....................................... 48

  Morphology of Inflammatory Cells .................................. 48
   Mononuclear Phagocyte .............................................. 48
   Eosinophilic Granulocyte .......................................... 50
   Heterophilic Granulocyte .......................................... 51
   Small Mononuclear Cell (Lymphocyte) ............................ 52
   Plasma Cell .......................................................... 52

  Histochemistry ...................................................... 52

  Ultrastructure of Splenic and Renal Macrophage Aggregates ... 53

**DISCUSSION** ....................................................... 54

**FIGURES** .................................................................. 63

**LITERATURE CITED** ................................................. 77

CHAPTER 4: THE EFFECTS OF CADMIUM CHLORIDE ON DEVELOPMENT OF EXPERIMENTALLY INDUCED HEPATIC MACROPHAGE AGGREGATES AND GRANULOMAS IN *RIVULUS MARMORATUS* ............................................. 84

**INTRODUCTION** .................................................... 84

**MATERIALS AND METHODS** ........................................ 86
LIST OF FIGURES

Figure 2.1: Gross and microscopic tissue changes in liver of *Rivulus marmoratus* experimentally infected with *Calyptospora funduli* ............................................... 35

Figure 2.2: Cellular response to stages of *Calyptospora funduli* in liver of experimentally infected *Rivulus marmoratus* .......................................................... 37

Figure 2.3: Macrophage aggregate and granuloma development in liver of *Rivulus marmoratus* in response to *Calyptospora funduli* infection. ............................. 39

Figure 3.1: Morphogenesis of hepatic macrophage aggregates in *Rivulus marmoratus* infected with *Calyptospora funduli*; 8-15 days p.i. .................................................... 64

Figure 3.2: Morphogenesis of hepatic macrophage aggregates in *Rivulus marmoratus* infected with *Calyptospora funduli*; 18-40 days p.i. ..................................................... 66

Figure 3.3: Morphogenesis of hepatic macrophage aggregates in *Rivulus marmoratus* infected with *Calyptospora funduli*; 60-150 days p.i. .................................................... 68

Figure 3.4: Sequential changes in the mononuclear phagocyte of *Rivulus marmoratus* during formation of hepatic macrophage aggregates. ............................. 70

Figure 3.5: Host leukocytes associated with hepatic macrophage aggregate formation in *Rivulus marmoratus*. ..................................................... 73

Figure 3.6: Splenic and renal macrophage aggregate ultrastructure in *Rivulus marmoratus*. ..................................................... 75
Figure 4.1: Measured aqueous cadmium concentrations (total) in water during exposure of *Rivulus marmoratus* infected with *Calyptospora funduli.*
Table 3.1: Histochemical characteristics of hepatic macrophage aggregates in Rivulus marmoratus experimentally infected with the coccidium Calyptospora funduli. ................................. 76

Table 4.1: Test concentrations and mortality data for Rivulus marmoratus exposed to CdCl₂·2.5H₂O in synthetic sea water (15 ppt, 24°C, pH = 8.4). ........................................... 97

Table 4.2: Morphometric comparison of macrophage aggregates (MA), granulomas (GR), and parasitic involvement (Oo) in cadmium-exposed (250 ppb) and unexposed Rivulus marmoratus experimentally infected with Calyptospora funduli. ................................. 98
ABSTRACT

Calyptradospora funduli is a pathogenic coccidium that infects the liver and pancreas of estuarine killifishes belonging to the genus Fundulus. Experimental infection of the atypical fish host Rivulus marmoratus with this intracellular protozoan parasite caused the development of hepatic macrophage aggregates (MA’s) and granulomas. The Rivulus/Calyptradospora host-parasite system thus represents a new laboratory model for investigations of MA biology. In the present study, this model was used to: 1) examine the morphology and sequential development of the parasite-elicited hepatic MA’s in R. marmoratus, 2) define the role that these structures play in the pathogenesis of an infectious disease process, and 3) quantify effects of a selected chemical toxicant on MA, granuloma, and parasite development using morphometric analysis. Histopathological evaluation of R. marmoratus at various time intervals following experimental infection indicates that parasite-elicited hepatic MA’s and granulomas represent endstages of a chronic inflammatory process in which a mononuclear phagocyte, apparently derived from the peripheral circulation, plays a prominent role. MA formation is initiated during the gamogonic development of the parasite in response to degenerating gamonts and necrosis of hepatocytes. Granulomas are elicited during sporogony and develop in response to the oocyst stage of the parasite. Sequential histochemical analysis of developing MA’s indicates that most of the pigment that is sequestered by these structures is the lipogenic substance ceroid. Melanin is present but not abundant. Ultrastructural analysis of parasite-elicited hepatic MA’s suggests that the sequestered pigments are lysosomally derived. Pigment granules are lysosomal residual bodies that are retained by macrophages after the intracellular digestion of phagocytosed host and parasite-derived debris. In addition to the mononuclear phagocyte, five other morphologically distinct leukocytes
are associated with the inflammatory response leading to MA and granuloma formation. Ultrastructurally, the parasite-elicited hepatic MA's resemble those that occur in the spleen and kidney of this species. Exposure of experimentally infected fish to cadmium chloride for 50 days suppressed parasite development and caused a reduction in the number and size of the MA's and granulomas. Cadmium did not measurably affect macrophage function at the tested exposure concentration.
CHAPTER 1: INTRODUCTION

Macrophages containing variable quantities of yellow-brown to black pigment granules are a frequent observation during the histological evaluation of fishes. In the Chondrichthyes and some primitive bony fishes, these macrophages are randomly distributed within certain tissues and organs; whereas in the higher Teleostei, they form discrete focal aggregations that have been termed melanin-macrophage centers (Roberts, 1975), hemosiderin bodies (Roales and Perlmutter, 1980), pigment nodules (Jolly, 1923; Fulop and McMillan, 1984), pigmented mictic bodies (Wolke et al., 1985a), and recently, macrophage aggregates (MA’s) (Brown and George, 1985; Wolke et al., 1985b). These structures sequester at least three types of pigment and occur frequently, but not exclusively, in the teleost spleen, kidney, and liver. Functions attributed to MA’s include a primary role in tissue catabolic processes, clearance of soluble and particulate antigens from circulation, immune modulation, and a role in the pathogenesis of various infectious and non-infectious diseases (Agius, 1985).

A critical examination of the scientific literature indicates that experimental induction of MA’s has not been achieved. Thus, biological investigations have been restricted to the study of those structures that already exist in the tissues of feral or experimental animals. Infection with the intracellular coccidian parasite Calyptospora funduli has been observed to elicit the formation of MA’s in the liver of the estuarine cyprinodontid teleost Rivulus marmoratus. In the present study, this host-parasite system was employed as an experimental model in order to examine the role of MA’s in the pathogenesis of this parasitic infection, and to examine morphological, functional, and morphogenetic aspects of the relationship between developing MA’s and this parasitic infection. Additionally, this host-parasite system was
used as an in vivo model to evaluate the effects of the heavy metal cadmium on macrophage function.

EARLY STUDIES

Early studies regarding the nature of the pigments sequestered by piscine macrophages are contradictory. Jolly (1923 as cited by Mackmull and Michels, 1932) was probably the first to describe pigment-bearing macrophages in teleosts. He considered aggregates of these cells in the kidney to represent sites where effete erythrocytes were destroyed. Yoffey (1929) noted aggregates of pigmented macrophages in teleost spleens and called them pigment nodules. Mackmull and Michels (1932) demonstrated that in the marine labrid fish Tautogolabrus adspersus, intraperitoneally injected carbon was ultimately sequestered by splenic and renal aggregations of macrophages. Wood and Yasutake (1956) observed pigment that they considered to be ceroid in the phagocytic and parenchymal cells of fish that had been exposed to toxic materials and of fish with microbial infections and nutritional deficiencies. Similarly, histochemical evaluation indicated that the pigments sequestered by splenic, renal, and hepatic aggregates in the freshwater teleosts Cyprinus carpio, Pimelodus maculatus, and Prochilodus scrofa were derived predominantly from lipids (Tokumaru and Ferri, 1970). These authors concluded that the bulk of this pigment was ceroid; however, they also demonstrated the presence of ferric iron. In contrast, studies with the rainbow trout, Onchorhynchus mykiss, indicated that in the renal tissues of this salmonid, the sequestered pigments were predominantly melanin (Roberts, 1975; Oguri, 1976). Roberts (1975) first called these cells "melanin-macrophages" and the focal aggregates that they form in tissues "melanin-macrophage centers". Adopting Edelstein’s (1971) definition of melanin, Roberts suggested that the yellowish-brown to blackish pigments observed in these structures were related, and represented temporal stages in the
oxidation of indigestible lipid residues culminating in the formation of lipofuscin (Edelstein's liponeuromelanin). He hypothesized that the lighter colored pigments (ceroid) observed in some macrophages represented an early stage in the development of the black pigments (melanin) observed in others. Roberts (1975) considered the "melanin-macrophage" to be an important component of the piscine reticuloendothelial system.

MORPHOLOGY OF MACROPHAGE AGGREGATES

Morphological studies have focused predominantly on MA's of the spleen and kidney. Histologically, these structures are nodular and usually surrounded by a delicate, argyrophilic fibro-cellular capsule (Roberts, 1975; Fulop and McMillan, 1984; Agius, 1985; Herraez and Zapata, 1986). They are quite variable in size, ranging from 40 to 1000 μm in size (Wolke et al., 1985a). Splenic MA's in hybrid sunfishes (Lepomis spp.) range from 12 μm to over 230 μm in diameter (Fulop and McMillan, 1984). In the turbot, Scophthalmus maximus, bluegill, Lepomis macrochirus, and goldfish, Carassius auratus, splenic MA’s are often located adjacent to blood vessels and ellipsoids, and may be bordered by a prominent capsule of lymphoid cells (Roberts, 1975; Agius, 1985; Herraez and Zapata, 1986). Renal MA’s are often associated with peritubular capillaries and vascular sinuses, whereas in the liver these structures often occur in close proximity to the hepatic blood vessels. The constituent macrophages are replete with refractile pigment granules that are heterogeneous in size and shape, and yellow-brown to black in hematoxylin and eosin-stained histologic sections (Roberts, 1975).

Histochemically, the pigments of MA’s are acid-fast, periodic acid-Schiff (PAS) positive, Schmorl’s positive, Sudan black positive, and DOPA-negative. In the spleen they are additionally positive with Perl's stain for ferric iron (Tokumaru and Ferri, 1970; Roberts, 1975; Kranz and Peters, 1984; Agius, 1985). Agius and Couchman (1986)
demonstrated enhanced alkaline phosphatase activity in MA’s of the tilapia, Oreochromis aureus following prolonged starvation, and starvation that was followed by antigenic stimulation with heat-killed Aeromonas hydrophila. Splenic MA’s of hybrid sunfish were PAS-, Sudan black-, and weakly acid phosphatase-positive, stained with dilute basic dyes such as neutral red and methyl green, and were positive with the Schmorl’s ferricyanide and Gomori’s Prussian blue methods for ferric iron (Fulop and McMillan, 1984). In the goldfish, Carassius auratus, histochemical analyses of splenic, renal, and hepatic MA’s demonstrated the presence of lipids, neutral mucopolysaccharides, and traces of basic proteins. Lipofuscin was demonstrated by the Schmorl’s method but not with the more selective Nile blue reagent (Herraez and Zapata, 1986). Splenic MA’s were strongly positive for melanin; whereas, renal MA’s stained weakly. Tests for acid phosphatase activity showed slight reactivity in some MA’s; whereas tests for alkaline phosphatase and endogenous peroxidase were negative. In contrast to the observations of other investigators (i.e. Tokumaru and Ferri, 1970; Roberts, 1975; Agius, 1979; Kranz and Peters, 1984) MA’s in the liver of the goldfish were also positive with Perl’s stain for ferric iron (Herraez and Zapata, 1986). Similarly, Myers et al. (1987) observed that hepatic MA’s in the English sole, Parophrys vetulus, from chemically contaminated environments in Puget Sound, Washington, were strongly positive for ferric iron.

Ultrastructurally, the cells comprising MA’s are complex. Agius and Agbede (1984) examined the MA’s of teleost fishes. Constituent macrophages were replete with heterogeneous inclusions identified as lipofuscin, melanin, and hemosiderin granules as well as degenerating cellular organelles. These materials were usually associated with the lysosomal system, being located within secondary lysosomes and large phagolysosomes. Lipofuscin, the predominant pigment, was thought to be derived from damaged cellular components, such as mitochondria, through
the peroxidation of unsaturated fatty acids (Agius and Agbede, 1984). Hemosiderin was largely restricted to splenic MA’s and derived from the phagocytosis of effete erythrocytes. Melanin, present as groups of granules, as individual granules within phagolysosomes, or free in the cytoplasm, also appeared to be phagocytosed. Fulop and McMillan (1984) examined splenic MA’s of hybrid sunfish (Lepomis spp.) histologically and ultrastructurally. In this hybrid, the MA’s were enclosed by a fine capsule composed of reticular cells and a network of circumferentially arranged reticular fibers. Plasma cells and macrophages were often observed at the periphery of these structures. Macrophages comprising these MA’s were tightly apposed and contained heterogeneous, membrane-bound, cytoplasmic inclusions. These inclusions consisted of granular, electron-dense material representing erythrocytes in various stages of degradation, lysosomes, residual bodies, and other darkly stained debris, much of it recognizable as clusters of ferritin particles and complex siderosomes. In the river lamprey, Lampetra fluviatilis, the melanin-containing macrophages did not form aggregates as they do in the teleosts (Rowley and Page, 1985). They occurred in greatest numbers in the supraneural organ and in the cavernous bodies of the gills, where they were thought to constitute a first line of defense against microbial invasion. Ultrastructurally, these cells were complex, containing electron-dense granular inclusions resembling melanin granules, many of which were located within secondary lysosomes, phagolysosomes, and residual bodies or were free within the cytoplasm. Other than by the incorporation of melanin inclusions, the melanin-containing macrophages differed very little from the normal tissue macrophages. These cells contained a moderate amount of RER, a Golgi complex, and many primary lysosomes. A large number of pinocytotic vesicles often occurred in the peripheral cytoplasm. In the goldfish, Carassius auratus, splenic MA’s consisted of phagocytic cells within a network of reticular cells joined by prominent desmosomal attachments.
These reticular cells also formed an incomplete capsule around the MA's. Constituent macrophages had eccentric nuclei and their cytoplasm was completely filled with cellular debris. Cellular contents were heterogeneous, composed of crystalline inclusions, lipofuscin, lipid deposits, melanin granules, and remnants of granulocytes and erythrocytes that had been phagocytosed (Herraez and Zapata, 1986).

**PHYLOGENETIC AND ONTOGENETIC RELATIONSHIPS**

Agius (1980) conducted a histological survey of 72 species of fish representing the Agnatha, Chondrichthyes, and Osteichthyes and observed macrophages containing pigment in the hemopoietic tissues of all but the river lamprey, *Lampetra fluviatilis*, a species that was subsequently demonstrated to have these cells as well (Rowley and Page, 1985). An evolutionary trend was observed in both the distribution and degree of organization of melanin-containing macrophages (Agius, 1980). Whereas in Agnatha, Chondrichthyes, and the primitive Salmonidae individual melanin-containing macrophages were randomly distributed, in higher teleosts these cells formed organized aggregates. A shift in organ location from predominantly the liver in Agnatha, Chondrichthyes, and primitive bony fishes to the primary hemopoietic organs (spleen and kidney) in advanced teleosts suggested to Agius (1980) that MA's were lymphatic in nature, probably representing the primitive analogue of the avian and mammalian germinal center.

Pigment-bearing macrophages first appeared within the tissues of fish larvae shortly after their first feeding and then steadily accumulated with age, even in clinically normal individuals. Their presence without exception in older fish suggested that at least a portion of the sequestered pigments represented age or "wear and tear" pigment (Agius, 1981b). Brown and George (1985) conducted a quantitative study of the relationship between pigment content in the anterior kidney and age, sex, condition index, and degree of parasitic
infection in the yellow perch, *Perca flavescens*. Of the four variables examined, only age correlated positively with renal MA quantity, suggesting that pigment accumulation in this instance was not pathological but represented the normal aging process.

**MACROPHAGE AGGREGATE FUNCTIONS**

**Tissue Catabolism**

Several investigations indicate that MA's play a role in tissue catabolic processes. Some investigators consider splenic MA's to be involved predominantly in phagocytosis and degradation of effete erythrocytes and the subsequent recycling of ferric iron (Graf and Schlüns, 1979; Fulop and McMillan, 1984; Kranz and Peters, 1984). Agius (1979) examined splenic, renal, and hepatic MA's histochemically for the presence of hemosiderin. In 14 teleost species that he examined, only the splenic MA's consistently sequestered iron. Both in fish subjected to prolonged starvation and in fish with infections that induced a hemolytic anemia, Agius (1979) observed an increase in the storage of iron by splenic MA's; however, he did not observe iron deposited in the liver or kidney. In contrast, even after prolonged starvation of the dogfish, *Scyliorhinus canicula*, hemosiderin was not stored by MA's in any organ (Agius, 1983). Agius (1981a) was able to demonstrate an increase in the storage of hemosiderin in the kidney of the rainbow trout, *Onchorhynchus mykiss*, following splenectomy and subsequent starvation. Agius and Roberts (1981) quantified the effects of starvation on splenic, renal, and hepatic MA's of five fish species. These investigators observed a net increase in the number and size of MA's with time, suggesting that catabolic processes contributed to the formation of pigments sequestered by these structures and that pigment deposition was initiated by the extensive tissue degeneration elicited during starvation.
**Immunological Studies**

Several studies suggest that MA's modulate immune functions in fishes. The observation by Mackmull and Michels (1932) that in the cunner intraperitoneally injected inert materials such as colloidal carbon are ultimately transported to and sequestered by MA's has been verified in other species including the plaice, *Pleuronectes platessa*, by Ellis et al. (1976); goldfish, *Carassius auratus*, by Mori (1980) and Herraez and Zapata (1986); rainbow trout, *Onchorhynchus mykiss*, by Tatner and Manning (1985); carp, *Cyprinus carpio*, and rosie barb, *Barbus conchonius*, by Lamers and Parmentier (1985); and river lamprey, *Lampetra fluviatilis*, by Page and Rowley (1984). Similarly, particulate antigens (Ag) such as sheep erythrocytes and bacterins (Secombes and Manning, 1980; Lamers and Pilarczyk, 1982; Maas and Bootsma, 1982; MacArthur et al., 1983; Lamers and De Haas, 1985; Lamers, 1986; Herraez and Zapata, 1986; 1987) and soluble Ag such as bovine serum albumin (BSA) and human gamma globulin (HGG) (Ellis, 1980; Secombes and Manning, 1980) are also sequestered by splenic and renal MA's. Further evidence that MA's modulate immune functions was provided by Ellis and de Sousa (1974), who demonstrated that in the plaice a population of radiolabeled lymphocytes migrated to and localized within the white pulp surrounding splenic and renal MA’s. Similarly, Roberts (1975) observed a prominent capsule of lymphoid cells associated with splenic and renal MA’s in certain species.

Lamers and De Haas (1985) reported that Ag processing in the carp was similar to antigen processing in mammals. *Aeromonas hydrophila* Ag injected IM into carp was initially immobilized and phagocytosed by macrophages of the splenic ellipsoids and by solitary phagocytic cells of both the splenic pulp and renal hemopoietic tissues. However, by day 15 post-inoculation this Ag became localized in and around the MA’s, mainly on the outer surface of cells where it was retained for up to 12 months. Localization of *A. hydrophila* Ag within MA’s coincided with the
formation of clusters of pyroninophilic cells in close proximity to MA's, localization of immunoglobulin on the outer surfaces of "melanomacrophages", and establishment of a peak in serum antibody titers. These observations suggested that Ag might be retained by MA's for long-term storage as immune complexes, and that these structures might represent a micro-environment for the antigenic stimulation of immunocompetent lymphoid cells, much as it occurs in avian and mammalian lymph nodes (Lamers, 1986). Furthermore, the long-term retention of Ag by these structures was interpreted to imply a role in immunological memory (Lamers, 1986). These studies support the view of some investigators that MA's represent the primitive analogue of the mammalian germinal center (Ellis et al., 1976; Ferguson, 1976; Agius 1985). However, differences of opinion exist. Secombes et al. (1982) hypothesized that aggregates of pyroninophilic cells forming in the hemopoietic tissues of carp immunized with HGG or Aeromonas salmonicida antigen might represent the functional analogue of the homeotherm germinal center. Herraez and Zapata (1986) observed a net increase in the size and number of splenic and renal MA's in goldfish inoculated with SRBC's, a putative T-independent antigen; however, they found no correlation between antibody titers and increases in size or number of MA's. In contrast, inoculation of goldfish with formalin-killed Yersinia ruckeri, a T-independent antigen, caused no changes in number, size, or percentage area occupied by MA's (Herraez and Zapata, 1987).

Disease Associations

Macrophage aggregates are thought to participate in a variety of infectious disease processes. These structures have been observed to play a role in the pathogenesis of aeromonad infections that cause severe necrosis of the hemopoietic tissues in salmonid fishes (Thorpe and Roberts, 1972). Roberts (1975) considered MA's to be a prominent component of the piscine reticulo-endothelial system and, hence, part of
the defense system that protects fishes against microbial infections. This investigator reviewed the association between the melanin-containing macrophage and various viral, bacterial, and protozoal diseases, distinguishing between these cells and true melanocytes (melanin-synthesizing cells), which play a role in the cellular response to certain trematode infections of fish. Roberts related the sequestration of melanin by the piscine macrophage with the functions that have been proposed for this pigment. Melanin can function as a free radical scavenger, binding aromatic and cyclic compounds and cations; thus protecting the macrophage from these potentially toxic compounds and ions. Conversely, the free-radical characteristics of melanin and its precursors and the oxidation of melanin with the production of hydrogen peroxide are bactericidal properties and may augment the ability of this cell to kill microbial organisms (Edelstein, 1971). Roberts hypothesized that these attributes could explain the presence of pigment-bearing macrophages in various bacterial lesions of fish. This investigator also described an association between MA's and chronic skin ulcers caused by bacterial infections, including those caused by *Vibrio anguillarum*. The pigment-bearing macrophages within these skin lesions were speculated to derive from splenic or renal MA’s. Roberts speculated that the macrophages within splenic and renal MA’s could actively migrate into the vasculature and thus be transported to sites of inflammation. In that same report, Roberts observed that the spores of the myxosporidan, *Myxobolus pseudodispar*, localized specifically within splenic and renal MA’s of the roach, *Rutilus rutilus*. Roberts (1976, cited by Agius, 1985) observed an association between melanin-bearing macrophages and lymphocystis disease in the plaice, *Pleuronectes platessa*. MA’s were reported to occur in association with granulomatous inflammatory lesions in the spleen, kidney, and liver of the scabbard fish, *Aphanopus carbo*, infected with an *Ichthyophonous*-like fungus (Agius, 1978). These structures also
occurred in the gills of Atlantic salmon, *Salmo salar*, infected with *Ichthyobodo* sp. (Ellis and Wootten, 1978). Huizinga et al. (1979) observed increased numbers of MA's in the kidney, liver, and spleen of the largemouth bass, *Micropterus salmoides*, afflicted with red sore disease. These structures were particularly abundant immediately surrounding trematode metacercariae.

Several investigators have described associations between coccidian parasites and and MA's. Ferguson (1976) observed architectural changes in splenic ellipsoids and MA's of the turbot, *Scophthalmus maximus*, infected with an undescribed coccidian parasite. He suggested that ellipsoidal macrophages replete with cellular debris of host or protozoal origin actively migrated to the MA's. Dyková and Lom (1981) described structures called "yellow bodies" associated with oocysts of *Goussia carpelli* (as *Eimeria carpelli*), *Eimeria acerinae*, *E. cheni*, and *E. sinensis*. Kent and Hedrick (1985) identified the yellow bodies in *G. carpelli* infections of *Carrassius auratus* histochemically and ultrastructurally as lipofuscin or ceroid within macrophages. Dyková et al. (1983) observed MA's in the gills of roach, *Rutilus rutilus*, infected with the coccidium, *E. branchiphila*. Molnar (1981) described pigment formation associated with the host response to several extra-intestinal coccidians including *Eimeria degiustii*, *E. metschnikovi*, *E. scardinii*, and *E. siliculiformes*. The sequestration of 100-600 oocysts into discrete foci was thought to be carried out by epithelioid macrophages. These cells contained abundant yellowish or brownish pigment and were thought to dissolve and destroy old oocysts. Solangi and Overstreet (1980) observed pigment deposition in the livers of the Gulf killifish, *Fundulus grandis*, infected with the coccidian, *Calyptospora funduli* (as *Eimeria funduli*). They considered this pigment to be lipofuscin, but did not observe it consistently in naturally infected cyprinodontid fish hosts. MA's have also been observed in association with metazoan infections (Overstreet and Thulin, 1989) and
in studies of experimental wound healing (Roberts et al., 1973; Vogelbein and Overstreet, 1987).

Pigment deposition within phagocytic and parenchymal cells has also been observed in nutritionally compromised fishes. Smith (1979) illustrated aggregates of ceroid-laden macrophages as well as hepatocytes containing ceroid in livers of rainbow trout fed a rancid diet. Similarly, Atlantic salmon fed a rancid diet developed a prominent hepatocellular ceroidosis (Roald et al., 1981). Blazer and Wolke (1983) described aggregates of ceroid-bearing macrophages in a variety of organs of the cultured clownfish *Amphiprion ocellaris* maintained at elevated water temperatures and fed a rancid, vitamin A- and E-deficient diet.

**Toxicologic Associations**

Macrophage aggregates have been proposed as sensitive but nonspecific histological indicators of physiological stress and environmental quality (Agius, 1985; Wolke et al., 1985a,b; Blazer et al., 1987). These structures are elevated in number and size in the tissues of fishes inhabiting chemically polluted environments. Hepatic MA's in the English sole, *Platichthys stellatus*, and the starry flounder, *Parophrys vetulus*, from the heavily polluted Duwamish River in Puget Sound, Washington, were more abundant and larger than those in fish from relatively unpolluted habitats (Pierce et al., 1978; 1980; Myers et al., 1987). Similarly, Haensly et al. (1982) found that one significant long-term effect of the "Amoco Cadiz" oil spill off the coast of France was an increase in the abundance of hepatic MA's in the plaice, *Pleuronectes platessa*, inhabiting the most severely contaminated areas. Similar associations have been described in the spleen and liver of the ruffe, *Gymnocephalus cernua*, flounder, *Platichthyes flesus*, and smelt, *Osmerus eperlanus*, inhabiting the polluted Elbe estuary in Germany (Kranz and Peters, 1984; Peters et al., 1987) and in the sand...
flathead, *Platypocephalus bassensis*, and globefish, *Diodon hystrix*, inhabiting Port Phillip Bay, a heavily contaminated estuary in Tasmania (Langdon, 1986). Kranz and Peters (1984) hypothesized that hepatic MA’s of ruffe inhabiting polluted habitats may concentrate toxic chemicals, thus eliminating them from metabolic systems. The MA’s in many of these studies were associated with various toxicogenic lesions including neoplastic tissue alterations. Macrophages laden with hemosiderin and ceroid occurred in the livers of pen-reared Atlantic salmon exhibiting a toxicopathic liver disease of uncertain etiology. This liver disease was characterized by diffuse hydropic degeneration of hepatocytes, severe hepatocellular necrosis, and hepatic megalocytosis. It was thought to be caused by exposure of the fish to an unidentified natural toxin (Kent et al., 1988).

Several attempts have been made to quantify the effects of environmental variables on certain MA parameters. Selected MA parameters were compared in the winter flounder, *Pseudopleuronectes americanus*, from chemically polluted versus clean sites (Wolke et al., 1985b; Lloyd, 1988) and in the largemouth bass, *Micropterus salmoides*, from thermally affected and unaffected sites (Blazer et al., 1987). These studies demonstrated significant increases in size and number of MA’s in fish from the degraded environments; however, age, relative weight, and season of capture have also been observed to affect the number of MA’s and the content and distribution of pigments within them (Brown and George, 1985). Benyi et al. (1989) and Gardner et al. (1989) observed that the tissue section area occupied by splenic MA’s increased in winter flounder with increasing levels of benzo[a]pyrene sediment contamination. Furthermore, in spleens of flounders exhibiting hepatic neoplasms, the area occupied by MA’s was greater than in fish without neoplasms. These studies support the view that MA’s might serve as sensitive but non-specific histological indicators of fish health and environmental quality (Wolke et al., 1985b; Blazer et al., 1987).
Several investigations report either no effect or actual declines in both MA number and size following toxicant exposure. Laboratory exposure of the plaice, *Pleuronectes platessa*, to potassium dichromate caused a three-fold increase in the number of splenic MA's; yet, because of a concomitant three-fold decrease in MA size, the percentage area occupied by these structures remained the same (Kranz and Gercken, 1987). The dab, *Limanda limanda*, from heavily contaminated sites in the German Bight exhibited fewer and smaller splenic MA's than dab from less contaminated sites (Kranz, 1988). Similarly, winter flounder, *Pseudopleuronectes americanus*, exposed to crude oil exhibited a decline in the number of hepatic MA's with increasing oil concentrations (Payne and Fancey, 1989). Cunner, *Tautogolabrus adspersus*, exposed to high concentrations of cadmium chloride exhibited a reduction in the hemosiderin content of renal MA's (Newman and MacLean, 1974). Reductions in the number, size, and pigment content of MA's in these studies were attributed to immune suppression, presumably the suppression of macrophage function caused by exposure to these chemical toxicants.

RATIONAL AND HYPOTHESES OF THE STUDY

The disagreement among investigators regarding proper terminology for aggregates of pigment-bearing macrophages in fishes and the many conflicting reports encountered in the literature suggest that the fundamental nature of these structures is still poorly understood. Although MA's of the spleen and kidney have been studied extensively, the MA's occurring in other organs and tissues have not been adequately examined. That splenic MA's degrade effete erythrocytes and play a role in the subsequent storage and recycling of iron is now widely accepted. Similarly, the role of splenic and renal MA's in the clearance of particulate and soluble antigens from the blood stream has also been adequately demonstrated. However, MA's may also develop in response to localized stimuli within other tissues and organs. Brown and George
(1985) suggested that these structures may arise as a result of the normal aging process or as a consequence of varied pathological processes. Yet, in many instances, identifying the underlying causes of MA formation is not possible. This is especially true for the MA's that occur in liver. Since a model that permits experimental production of MA's is not available, the morphogenesis of these structures has never been examined in fishes. Questions remain about the kinetics of their formation. The speed with which MA's form and how long these structures persist is poorly understood, and little is known about the relationship between pigment-bearing macrophages and other mononuclear phagocytes (monocytes, macrophages) of fishes. Similarly, the nature and origin of the sequestered pigments have been inferred largely from morphological studies of existing MA's and from their histochemical and ultrastructural resemblance to similar pigments occurring in mammalian tissues. Finally, few studies have specifically examined the relationship between MA's and infectious disease agents, even though these structures are frequently observed in association with infections. Thus, the potential role that MA's play in the pathogenesis of certain infectious diseases is poorly understood. To address these aspects of MA biology, an appropriate model that would permit the experimental induction of these structures is required.

**Calyptospora funduli** is a pathogenic coccidium that infects the liver and pancreas of estuarine killifishes belonging to the genus *Fundulus*. In heavily infected individuals, up to 85% of those organs may be replaced by oocysts (Solangi and Overstreet, 1980). An unusual feature of this parasite is its requirement for an invertebrate intermediate host (Solangi and Overstreet, 1980; Fournie and Overstreet, 1983). Transmission of the infection requires that an infective stage developing in the alimentary tract of the grass shrimp, *Palaemonetes pugio*, be eaten by the fish host (Fournie and Overstreet, 1983). The ability to induce experimental infections in fish has facilitated
investigations of parasite development (Hawkins et al., 1983a,b,c, 1984a) and host-parasite interactions (Hawkins et al., 1984b). It has also permitted morphological studies of the inflammatory response elicited by the parasite in its natural definitive fish hosts (Hawkins et al., 1981). In the natural fish hosts, initiation of an intense but transient leukocytic response coincided with the appearance of the parasite's sexual stages during gamogony. This cellular response peaked during early sporogony and terminated with a decline in leukocyte influx and fibrotic encapsulation of oocyst aggregates occurring about 30 days post-infection (Solangi and Overstreet, 1980; Hawkins et al., 1981). Although not a consistent observation, yellow to brown pigment deposits identified histochemically as lipofuscin and melanin were associated with encapsulated oocyst aggregates in Fundulus grandis and F. similis (Solangi and Overstreet, 1980). Fournie (1985) examined the host response to C. funduli in a variety of species that normally do not serve as definitive hosts for this parasite. In the estuarine cyprinodontid Rivulus marmoratus, the initial acute leukocytic response did not subside during sporogony as in the natural fish hosts, but rather it progressed to a chronic cellular response that culminated with the granulomatous encapsulation of oocysts and hepatic MA formation. This atypical host-parasite relationship thus appears to be a good model system to examine the role that MA's play in an infectious disease process. Since most stages of C. funduli develop synchronously within individual fish and also within groups of fish experimentally infected at the same time (Solangi and Overstreet, 1980), this system lends itself to sequential studies of MA formation.

The following hypotheses were formulated to define the role that MA's play in the pathogenesis of this parasitic infection and to clarify temporal aspects of their development:
1. The hepatic MA’s that develop in association with *Calyptospora funduli* infections in *Rivulus marmoratus* represent the outcome of a local chronic inflammatory response elicited by degenerating stages of the parasite.

2. The predominant cell type involved in formation of parasite-induced hepatic MA’s in *R. marmoratus* is a mononuclear phagocyte derived from the host blood circulation.

3. The bulk of pigment sequestered by hepatic MA’s in infected *R. marmoratus* is ceroid and is derived from the intra-cellular digestion of host cellular and parasite debris phagocytosed by mononuclear phagocytes during the initial stages of an inflammatory process.

One aspect of MA biology that remains controversial is the application of these structures as indicators of physiological stress and environmental health. Although specific causal factors have not been identified, fluctuations (increases or declines) in MA size and number in fishes inhabiting chemically contaminated environments have been attributed to increases in tissue catabolic processes or suppression of immune functions, specifically macrophage activity. A model of MA formation that can be manipulated in the laboratory would be useful to identify specific environmental factors that might influence the abundance of MA’s in feral fishes. The following hypothesis was formulated to evaluate the potential application of the *Rivulus-Calyptospora* model to evaluations of the effects of toxicant exposure on the development of MA’s.

4. Exposure of experimentally infected *R. marmoratus* to cadmium chloride suppresses the development of hepatic MA’s.
To test hypotheses 1-3, fish were experimentally infected and subsampled at various times post-infection. Results of a histological evaluation of hepatic MA formation are presented in Chapter II. The results of detailed histochemical and ultrastructural analyses of hepatic MA formation, morphologic description of the inflammatory cell types associated with MA formation, and morphological comparisons of the experimentally induced hepatic MA's with those in the spleen and kidney of *R. marmoratus* are outlined in Chapter III. Chapter IV describes the results of a study designed to quantitatively evaluate the effects of cadmium chloride-exposure on the development of hepatic MA's in experimentally infected rivulus.

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ABSTRACT: Formation of hepatic macrophage aggregates (MA’s) in *Rivulus marmoratus* was induced with the coccidian parasite *Calyptospora funduli*. Experimental infections were produced by feeding infected intestine and hepatopancreas from grass shrimp (*Palaemonetes pugio*) to individual parasite-free fish. Livers of fish sampled 5-150 days post-infection were examined histologically. Mild diffuse inflammation initiated during early merogony (5-8 days), consisted of eosinophilic granulocytes and heterophils. Liberation of merozoites (8-12 days) caused extensive hepatocyte degeneration and augmented leucocyte exudation. During gamogony (15-18 days) mononuclear phagocytes became a predominant component of the cellular exudate. Focal lesions, considered to be early MA’s, became apparent during early sporogony (20-25 days); they consisted of degenerating infected hepatocytes and inflammatory cells, and contained developing oocysts and degenerating macrogamonts, but no pigment. Early MA’s became visible grossly at 30 days as diffuse yellow-tan foci within the hepatic parenchyma. Pigment content increased progressively, but melanin was never a major component. Oocysts first elicited a granulomatous response between 40 and 50 days. The study suggests that MA formation in this instance is an inflammatory process in which mononuclear phagocytes recruited from peripheral circulation play a dominant role. MA development appears to be elicited by macrogamont degeneration, whereas granuloma formation is probably a response to oocysts.
Pigment-bearing macrophages are a prominent feature of piscine haemopoietic tissues (Agius, 1985). In the higher teleosts, these cells form aggregates that occur most frequently in the spleen, kidney and liver. Roberts (1975) called these structures melano-macrophage centers. Recently, however, they have also been called macrophage aggregates (MA's) (Brown & George, 1985). Four pigments may be present: haemosiderin, ceroid, lipofuscin and melanin. The lipogenic pigments are generally the most abundant (Agius & Agbede, 1984).

Functions ascribed to MA's include iron storage following erythrophagocytosis (Agius, 1979, 1981), clearance of soluble and particulate materials from circulation (Ellis, 1980; Rowley & Page, 1985), antigen-processing in immune responses (Ellis & de Sousa, 1974; Agius, 1985; Lamers & DeHaas, 1985) and tissue catabolism as indicated by starvation experiments (Agius & Roberts, 1981). These studies suggest that the general function of the MA is centralization of endogenous and exogenous materials for destruction, detoxification, or recycling.

Macrophage aggregates occur in association with a variety of infectious diseases, including lymphocystis (Roberts, 1976) and infections caused by Ichthyophonus (Agius, 1978), Ichthyobodo (Ellis & Wootten, 1978) and Myxobolus pseudodispar (Roberts, 1975). Associations between extra-intestinal coccidian parasites and MA's have been reported (Solangi & Overstreet, 1980; Molnár, 1981; Paterson & Desser, 1982). Nevertheless, the sequential development of MA's and their role in ongoing infectious disease processes have not been documented. The cyprinodontid fish Rivulus marmoratus infected with the coccidium Calyptospora funduli serves as a good model for this documentation. Our purpose was to describe and assess hepatic MA formation by investigating a special case of inflammation in response to
C. funduli in the atypical host R. marmoratus.

II. MATERIALS AND METHODS

Adult, laboratory-reared specimens of R. marmoratus were used in this experiment. One hundred fish were experimentally infected with C. funduli according to the method described by Fournie & Overstreet (1983). Grass shrimp (Palaemonetes pugio) were individually fed liver from wild Fundulus grandis heavily infected with sporulated oocysts of C. funduli for a 3-day period and then maintained in a 55-l aquarium for 7 days. Rivulus marmoratus was infected with C. funduli by feeding individuals the alimentary tract and hepatopancreas of experimentally infected grass shrimp. Infected fish were maintained in two 75-l aquaria. Temperature and salinity were maintained at 22°C and 15 ppt, respectively. Fish were fed 24-h-old Artemia salina on every third day, and supplemented intermittently with Kordon Stress Flakes. Aquaria were provided with biological sponge filters and with adequate shelter to minimize aggressive behaviour characteristic of R. marmoratus (Koenig & Chasar, 1984). Five fish were sampled on each of days 5, 8, 10, 12, 15, 18, 20, 25, 30, 40, 50, 60, 80, 100 and 150 post-exposure (p.i.). Twenty uninfected controls were examined, 10 on day 0 and 10 on day 150. Fish were anaesthetized individually with MS-222 (tricaine methanesulphonate). Liver, spleen and kidney were fixed for 2 h in 1.25% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M sodium cacodylate buffer (ph=7.4). Tissues were then dehydrated in a graded ethanol series and embedded in a glycol methacrylate embedding media (LKB Historesin). Blocks were sectioned at 2-3 μm on a Reichert Ultracut E ultramicrotome using glass knives. Sections were stained with haematoxylin and eosin-phloxine.
III. RESULTS

Endogenous development of *C. funduli* in *R. marmoratus* occurred primarily within hepatocytes. However, some parasites infected pancreatic acinar cells located within the liver. We observed two generations of asexual development (merogony). First generation meronts occurred in small numbers on day 5 p.i., maturing synchronously by day 8, on which day merozoites had been released in several fish. Second generation meronts developed during days 9-12 p.i. By day 15 p.i. differentiating gamonts occupied large portions of the hepatic parenchyma (gamogony). Immature developing oocysts first appeared on day 18 p.i., and by day 25 p.i. oocysts exhibited four developing sporoblasts (sporogony). Sporulation of oocysts was complete with resting-stage sporozoites on day 40 p.i.

*Calyptospora funduli* induced gross and microscopic changes within the hepatic parenchyma. Livers of experimentally infected fish sampled prior to 30 d p.i. and livers from uninfected controls were light tan to reddish brown in colour and contained almost no discrete pigment [Fig. 2.1(a)]. The livers of infected fish sampled on day 30 had a marbled surface [Fig. 2.1(b)] and contained diffuse pigmented foci quite variable in size and shape. Pigment in these developing MA’s was gold to light brown and always associated with nests of developing oocysts. All infected fish sampled subsequently had MA’s, often in large numbers. The pigments darkened progressively with time [Fig. 2.1(c)], and the MA’s became more discrete and compact [Fig. 2.1(d)]. Oocyst degeneration within MA’s increased progressively with time [Fig. 2.1(e)] and, after day 40 p.i., granuloma formation in response to oocysts occurred commonly [Fig. 2.1(f)].

*Calyptospora funduli* first elicited a leucocytic response during early merogony (5-8 days p.i.). This response was mild, diffuse and appeared not specifically directed toward developing meronts [Fig.
2.2(a)]. It consisted of eosinophilic granulocytes and heterophils loosely aggregated around hepatic blood vessels. Many eosinophils within or near necrotic foci were degranulating. Heterophils marginated and emigrated from many of the small hepatic blood vessels [Fig. 2.2(b)]. Liberation of first generation [Fig. 2.2(c)] and second generation merozoites caused hepatocyte degeneration and necrosis, and considerably augmented leucocyte exudation during days 8-12 p.i. During second generation merogony, large and small mononuclear cells occurred in increasing numbers within blood vessels and in oedematous tissue spaces [Fig. 2.2(d)]. The extent of inflammation increased during gamogony. Mononuclear cells became the predominant component of the leucocytic exudate. Eosinophils and heterophils continued to be recruited, and four leucocyte types occurred within and around the hepatic vasculature in large numbers [Fig. 2.2(e)]. During this period, the cellular response appeared for the first time to be directed specifically at the parasite. Macrogamonts, either as small groups or as individuals, became encapsulated by host leucocytes [Fig. 2.2(f)] and many showed signs of degeneration. Both granulocyte types and mononuclear cells participated in macrogamont encapsulation.

The leucocytic exudate first organized into discrete foci on day 20 p.i. These developing MA's consisted of loosely organized, poorly circumscribed cellular aggregates (probably macrophages). The cells contained degenerating macrogamonts [Fig. 2.3(a)] recognizable by their characteristic amylopectin granules. The surrounding extensive inflammatory exudate comprised a mixture of granulocytes and mononuclear cells. Monocytes and macrophages appeared to be the predominant components of this exudate. These early MA's contained viable developing oocysts which stained intensely basophilic; however, discrete pigment was not yet present.

During the subsequent 20-30-day period, developing MA's consolidated, became more discrete and circumscribed, and progressively
accumulated pigments. Melanin granules, although present, did not form a major component of these early MA's [Fig. 2.3(b)]. MA's consisted of pale tan cells containing degenerating gamonts and viable as well as degenerating oocysts. The MA's, located both within oedematous tissue spaces and parenchyma, became sequestered from normal hepatocytes by a delicate cellular capsule. A mixed leucocytic exudate, variable in intensity but comprised of granulocytes and mononuclear cells, surrounded many MA's [Fig. 2.3(b)].

Many degenerating as well as apparently viable oocysts elicited a granulomatous response starting c. 40-50 days p.i., especially when oocysts occurred at the periphery of MA's and when they were not associated with developing MA's [Fig. 2.3(c)]. In contrast, many oocysts within MA's did not elicit granuloma formation. During days 50-100 p.i. the response remained essentially the same. However, melanin granules and lipofuscin pigments progressively accumulated within MA's. After day 60 p.i., macrogamont-derived amylopectin granules could no longer be recognized histologically. Both granulomas and MA's continued to elicit a moderate leukocytic exudate. The mature MA's often continued to be cuffed by leucocytes, usually eosinophils and heterophils, even after 150 days p.i. [Fig. 2.3(d)]. Some intact oocysts remained, even after 150 days p.i.

IV. DISCUSSION

*Rivulus marmoratus* and *C. funduli* constitute an atypical host-parasite relationship. To our knowledge, this cyprinodontid fish is not a natural definitive host of *C. funduli*. Endogenous development of the parasite in experimental infections was abnormal and asynchronous. Many macrogamonts had become arrested in development and subsequently degenerated. Degeneration of oocysts included disruption and
dissolution of sporoblasts, sporonts and sporocysts. These changes seemed to increase progressively with time and were not regularly observed in prior experimental studies with estuarine species of Fundulus (Solangi & Overstreet, 1980; Upton & Duszynski, 1982; Hawkins et al., 1984).

The initial cellular response to C. funduli appeared similar in all host species studied thus far. It consisted of a mild, diffuse leucocytic infiltrate elicited during merogony (Solangi & Overstreet, 1980). In estuarine species of Fundulus, a heterophil responded first (Hawkins et al., 1981) whereas in R. marmoratus both eosinophilic and heterophilic granulocytes appeared first. During gamogony the cellular response was augmented considerably and appeared for the first time to be directed specifically at the parasite. Macrophages, lymphocytes, eosinophils and heterophils encapsulated gamonts in F. grandis (Solangi & Overstreet, 1980). Hawkins et al. (1981) suggested that augmented inflammation during gamogony probably resulted from the extensive damage to hepatocytes occurring during late merogony. In R. marmoratus, mononuclear phagocytes became a predominant component of the leucocytic exudate during gamogony. However, granulocytes initially encapsulated gamonts and may have caused their arrested development and subsequent degeneration. Although evidence of granulocyte-mediated cytotoxicity has been accumulating for a number of experimental mammalian host-parasite systems (e.g. Incani & McLaren, 1981; Yoshimura et al., 1984), functional aspects of piscine granulocytes remain largely unknown.

During sporogony, host responses to C. funduli in R. marmoratus and Fundulus spp. diverged drastically. In the natural definitive hosts (Fundulus spp.) the subsequent response was characterized by fibrosis. Filament-rich perisinusoidal cells and possibly fibroblasts deposited collagen and caused fibrotic encapsulation of developing oocysts (Hawkins et al., 1981). Following oocyst encapsulation, the leucocytic response in F. grandis subsided (Overstreet & Solangi, 1980).
Conversely, in *R. marmoratus*, fibrotic encapsulation of developing oocysts did not occur. The intense infiltration of mononuclear cells elicited during gamogony did not subside and ultimately led to hepatic MA and granuloma formation. Our results suggest that both MA's and granulomas were derived from monocytes recruited from peripheral circulation. This cellular derivation has been amply demonstrated in granuloma formation (Adams, 1983) but it may be a novel concept of MA origin. Pigment deposition has been associated with this protozoan infection (Solangi & Overstreet, 1980) but was never a consistent finding in the natural fish hosts. Increased parasite degeneration may have played a role in the consistent development of hepatic MA's in *R. marmoratus*. MA formation in this instance appears to be an inflammatory process, and these structures may in this case be viewed as a special type of granuloma. We have called them MA's, but do this with some reservations, realizing that functional differences may exist, depending on their location.

ACKNOWLEDGMENTS

We thank Dr. Will Davis of the Environmental Protection Agency, Gulf Breeze, Florida, for supplying laboratory-reared *R. marmoratus* and Cindy Dickens for typing the manuscript. This study was supported in part by Department of Commerce, NOAA, Mississippi-Alabama Sea Grant Consortium, under grant NA85AA-D-SG005 as publication MASGP-87-039, and National Marine Fisheries Service, under Pub. L. 88-309, Project No. 2-422-R.
Fig. 2.1: Gross and microscopic tissue changes in liver of *Rivulus marmoratus* experimentally infected with *Calyptospora funduli*. (a) On day 18 p.i.; x48. (b) Day 30, showing developing macrophage aggregates (MA's) (arrows); x45. (c) Squash preparation, day 150, showing MA's and oocysts (O) of *C. funduli*; x240. (d) Day 100, showing mature MA's (arrows); x42. (e) Squash preparation, day 80, showing degenerating oocysts (arrows) and pigment (P); x420. (f) Squash preparation, day 80, showing granuloma (G), degenerating oocyst (arrow) and pigment (P); x284.
Fig. 2.2: Cellular response to stages of *Calyptospora funduli* in liver of experimentally infected *Rivulus marmoratus*. (a) Minimal cellular response during early merogony, 8 days p.i., showing mature meronts (arrows); x804. (b) Section of blood vessel, 8 days p.i., with numerous marginating heterophils (arrows); x587. (c) Release of free merozoites (arrows), showing hepatocyte vacuolization and degeneration (DV); x720. (d) Aggregate of mononuclear cells (M) and eosinophils (E) within tissue space 10 days p.i. [second generation merogony, showing 2nd generation meronts (ME)]; x360. (e) Hepatic blood vessel 15 days p.i. (gamogony) containing heterophils (H) and mononuclear cells (M). Eosinophil (E), macrogamont (Ma), and microgamont (Mi); x583. (f) Host leucocytic encapsulation of gamonts 15 days p.i., illustrating heterophil (H), eosinophil (E), macrogamont (Ma) and microgamont (Mi); x660. All haematoxylin and eosin-phloxine.
Fig. 2.3: Macrophage aggregate and granuloma development in liver of *Rivulus marmoratus* in response to *Calyptospora funduli* infection. (a) Early MA 20 days p.i., containing degenerating gamonts (G), developing oocysts (O) and leucocytic exudate (arrows); x461. (b) Macrophage aggregate 50 days p.i., demonstrating pigment (P), sporulated oocysts (O), cellular capsule (C) and leucocytic exudate within oedematous tissue space (arrow); x368. (c) Mature epithelioid granuloma, 100 days p.i., sequestering oocysts (O); x240. (d) Mature macrophage aggregate 150 days p.i., with melanin granules (M) and oocysts (O), and surrounded by heterophils (H) and eosinophils (E); x386. All haematoxylin and eosin-phloxine.
REFERENCES


INTRODUCTION

A frequent observation during histologic evaluations of fishes is the presence of macrophages containing variable quantities of yellow-brown to black pigment granules (e.g., Agius 1985). In the higher teleosts, these macrophages form discrete focal aggregates that have been called melanin-macrophage centers (e.g., Roberts 1975), hemosiderin bodies (Roales & Perlmutter 1980), pigment nodules (Fulop & McMillan 1984), pigmented mictic bodies (Wolke et al. 1984), and recently, MA's (Brown & George 1985, Wolke et al. 1985). These structures occur most frequently, but not exclusively, in the spleen, kidney, and liver and sequester at least three different pigments (Agius 1985). Lipogenic pigments usually referred to as ceroid or lipofuscin generally comprise the bulk of this material; however, hemosiderin and melanin can also be sequestered in considerable amounts (Agius & Agbede 1984).

Several studies suggest that MA's are not restricted to the spleen, kidney, and liver. They have been observed in a variety of other tissues in association with infectious agents (Roberts 1975, 1976, Agius 1978, Ellis & Wootten 1978, Molnar 1981, Paterson & Desser 1982, Vogelbein et al. 1987, Overstreet & Thulin 1989), nutritional deficiencies (Smith 1979, Roald et al. 1981, Blazer & Wolke 1983), and degenerative and neoplastic conditions in fish from polluted environments (Pierce et al. 1980, Haensly et al. 1982, Kranz & Peters 1984, Malins et al. 1987, Myers et al. 1987, Peters et al. 1987, Vogelbein et al. 1990). This has prompted several investigators to suggest that an increase in the number and size of MA's may be a

An experimental method for eliciting synchronized MA formation has until recently been unavailable. Early studies have focused almost exclusively on existing aggregates of the spleen, kidney, or liver (for review see Agius 1985). To our knowledge, little is known concerning the morphogenesis of MA's and hence, the fundamental nature of these structures. We recently reported that experimental infections of the intracellular coccidian parasite *Calyptospora funduli* elicit formation of MA's in the liver of the cyprinodontid teleost *Rivulus marmoratus*. We used this atypical host-parasite system as a model to examine the histogenesis of these parasite-induced MA's (Vogelbein et al. 1987). In this study, we have used this host-parasite system to characterize ultrastructural and histochemical aspects of hepatic MA formation, to define the morphogenesis and nature of the pigments sequestered by these structures, and to ultrastructurally compare the parasite-elicited hepatic aggregates with those occurring in the kidney and spleen of this species. Additionally, we have assessed morphological aspects of the inflammatory cell types in *R. marmoratus* and their association with hepatic MA formation.

**MATERIALS AND METHODS**

The material examined in this study was subsampled for transmission electron microscopy (TEM) during a previous histopathological study of coccidian-induced MA formation (Vogelbein et al. 1987). That study provides details of the experimental design. Briefly, we sampled liver for TEM from 100 laboratory-reared adult *rivulus* (*Rivulus marmoratus*). These fish were experimentally infected
with *Calyptraspore funduli* according to the feeding method of Fournie & Overstreet (1983). Five fish were sampled on days 0, 5, 8, 10, 12, 15, 18, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, and 250 post-infection (p.i.), and the livers were processed for TEM by routine methods. Tissue blocks were fixed for 2 h in 3.0 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 0-4° C. This was followed by several buffer washes over a 6-h period, and post-fixation with 1.0 % OsO$_4$ in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature (22° C) for 2 h. Tissues were then dehydrated in a graded ethanol series, rinsed in propylene oxide, and embedded in Spurr's media (Spurr 1969). Representative tissue blocks from all 100 fish were semi-thin sectioned (1.0 μm) with glass knives and stained with methylene blue and azure B. Sections were screened light microscopically, and representative blocks were chosen for electron microscopic examination. Ultrathin sections were cut on either a Porter-Blum MT2B or a Reichert Ultracut E ultramicrotome using a Dupont diamond knife. Ultrathin sections were stained with lead citrate and uranyl acetate and examined on a Zeiss EM-10 or Jeol JEM-100SX transmission electron microscope. Images presented here generally represent those from fish with heavy experimental infections. Ultrastructural localization of peroxidase activity in the heterophil of *Rivulus marmoratus* was accomplished with the DAB technique described by Cannon et al. (1980) for the heterophil of the channel catfish *Ictalurus punctatus*.

Livers allocated for light microscopical evaluation were processed by routine methods, embedded in paraffin, and sectioned at 6 μm. In addition to routine hematoxylin and eosin staining, special histochemical methods included the Armed Forces Institute of Pathology (AFIP) method for lipofuscin, a Sudan black B stain for lipid-derived substances (ceroid), Schmorl's method for reducing substances (melanin), Perl's method for ferric iron, the periodic acid-Schiff (PAS) stain for
carbohydrates, and Gomori's stain for reticulum fibers (Luna 1968). These methods were applied to livers of fish sampled 0, 10, 20, 25, 30, 40, 50, 60, 70, 90, 125, and 150 d p.i.

RESULTS

Hepatic Macrophage Aggregate Formation

Infection by Calyptospora funduli elicited two morphologically distinct hepatic lesions in Rivulus marmoratus. Hepatic MA's developed in response to degenerating gamonts and tissue debris, and typical granulomatous lesions formed in response to the oocyst stages (also, see Vogelbein et al. 1987). We restrict this report to a description of MA formation. For details of parasite development and the tissue response in the typical definitive fish host, the reader is referred to Hawkins et al. (1981, 1984).

5-12 Days Post-Infection

The period between 5-12 d was characterized by intracellular asexual multiplication of the parasite (merogony), host cellular damage, and onset of an acute inflammatory response. Liberation of mature merozoites from infected host cells was accompanied by degeneration of damaged hepatocytes (Fig. 3.1a). An acute inflammatory response first occurred during this period with large numbers of host leukocytes aggregating within the vasculature and infiltrating the parenchyma (Fig. 3.1b). Leukocytes were largely confined to the immediate vicinity of hepatic blood vessels. This cellular response was mixed, consisting of three ultrastructurally distinct granulocytic leukocytes, which predominated at this time, and three agranulocytes. The ultrastructure of these cells is described below.
15 Days Post-Infection

By day 15, sexual stages of the parasite had formed (gamogony). Gamonts infected hepatocytes, and to a lesser degree, pancreatic acinar cells. Inflammation was extensive, and a mixed cellular exudate infiltrated and disrupted the parenchyma (Fig. 3.1c). Cords of inflammatory cells sequestered groups of infected hepatocytes; however, parasite and host cellular degeneration was rarely observed. Mononuclear cells resembling peripheral blood monocytes were the predominant component of the leukocytic infiltrate. Some of these cells, however, could be characterized as macrophages. They exhibited cytoplasmic rarefaction, a prominent Golgi complex, numerous dense bodies, an increase in rough endoplasmic reticulum (RER) and pinocytotic vesicles, and margination of the nuclear heterochromatin. They encapsulated the infrequent degenerating macrogamont (Fig. 3.1d). Evidence of endocytotic activity was rare; however, some macrophages contained small phagolysosomes (Fig. 3.1d).

18 Days Post-Infection

By 18 d p.i., many gamonts were degenerating, but early sporogonic stages continued to develop normally. Inflammation was diffuse, and host leukocytes, predominantly mononuclear cells, sequestered individual degenerating gamonts and hepatocytes (Fig. 3.2a). Phagocytically active macrophages had a complex cytoplasm including a prominent complement of lysosomes. Primary lysosomes were abundant and many phagocytes contained large, heterogeneous phagolysosomes. These structures were easily identifiable because many of them contained parasite-derived amylopectin granules (Fig. 3.2a). These granules were most prominent in the macrogamonts and appeared resistant to degradation by mononuclear phagocytes. Granulocytic leukocytes rarely exhibited evidence of phagocytosis.
20 Days Post-Infection

By 20 d p.i., poorly organized, discrete inflammatory lesions had formed. They consisted of phagocytically active macrophages and were encapsulated by host leukocytes. These loose cellular aggregates sequestered infected host cells, degenerating macrogamonts, and necrotic tissue debris (Fig. 3.2b). Many also contained viable parasites undergoing sporogony. Macrophages comprising these lesions contained prominent phagolysosomes, many of which retained amylopectin. The inflammatory infiltrate encapsulating these loose aggregates of macrophages was mixed, including three types of granulocytes and three types of mononuclear cells; however, monocytes and macrophages were the predominant cell types. These early lesions contained no grossly or microscopically recognizable pigments.

30-40 Days Post-Infection

Developing lesions became visible grossly by 30-40 d p.i. These tan to dark-brownish irregularly shaped foci were distributed randomly within the hepatic parenchyma (see Vogelbein et al. 1987). Constituent macrophages were more closely opposed than before but did not form intercellular junctions. Cell membranes of adjacent macrophages interdigitated slightly. Macrophage cytoplasm contained an abundance of primary lysosomes and large, morphologically heterogeneous phagolysosomes. Contents of some phagolysosomes resembled ceroid pigment. However, much of the endocytosed debris was still recognizably parasite-derived (Fig. 3.2c). Melanin granules first occurred within the developing lesions at this time. Host cells that contained melanin granules also often contained phagocytosed amylopectin granules (Fig. 3.2c). Melanosomes occurred individually within the cytoplasm, as well as in groups within phagolysosomes; however, this pigment was not a prominent component of the materials being sequestered within the developing lesions. Constituent cells at the periphery of developing
MA's generally sequestered less debris. These cells appeared less mature and resembled recently recruited monocytes and young inactive macrophages (Fig. 3.2d).

50-80 Days Post-Infection

Lesions during the 50-80 d period were well circumscribed and invested by a capsule of reticular cells. Some MA's had a thick cuff of leukocytes (Fig. 3.3a), whereas others had few such cells bordering their periphery. Constituent macrophages were filled with debris stored as large phagolysosomes or residual bodies, and amylopectin granules were recognizable in many cells. Intact macrogamonts were less abundant at this time, suggesting that they were being degraded within the MA's. Also, primary lysosomes were less abundant, indicating that macrophages were less actively digesting parasite debris. Melanin granules were abundant in some MA's and absent in others. Some MA's contained viable, fully sporulated oocysts (Fig. 3.3b).

100-250 Days Post-Infection

MA's at 100-250 d were discrete and compact. Some were encapsulated by a prominent cuff of leukocytes (Fig. 3.3c), whereas others were not (Fig. 3.3d). However, all MA's were bordered at the periphery by a thin capsule of reticular cells (Fig. 3.3d). At 150 d p.i. some MA's contained recognizable amylopectin granules (Fig. 3.3d), but intact gamonts were rare. Phagolysosomes of macrophages sequestered small groups of amylopectin granules (Fig. 3.3d). Melanin granules were prominent in some MA's, and many of these structures stored lipid droplets.

Morphology of Inflammatory Cells

Mononuclear Phagocyte
The mononuclear phagocyte underwent sequential morphological changes during formation and development of hepatic MA's. These changes corresponded temporally with parasite development. During merogony (8-12 d p.i.), the cellular inflammatory response contained mononuclear cells resembling the monocyte and macrophage (Fig. 3.4a). This cell averaged $8.5 \times 7.0 \, \mu m \,(N=50)$ in diameter, with a heterochromatic nucleus that exhibited indentations of the nuclear envelope. The cytoplasm contained prominent elongated mitochondria, a moderate amount of RER, and numerous free ribosomes (Fig. 3.4a). A prominent Golgi complex and associated membrane-bound dense bodies presumed to be lysosomes occurred in some cells (Fig. 3.4b). Occasionally, monocytes contained cytoplasmic lipid inclusions and microtubules.

During early sampling periods (12-18 d p.i.), mononuclear phagocytes occurred abundantly within hepatic blood vessels and within the perivascular parenchyma (Fig. 3.1b,c). However, they were randomly and loosely distributed, not aggregating at this time.

During gamogony (15-18 d p.i.), mononuclear phagocytes became the predominant inflammatory cell type. Many were enlarged (Fig. 3.4c), and contained numerous primary and secondary lysosomes. Some macrophages exhibited rarefaction of the cytoplasm. Nuclei were generally euchromatic, with chromatin marginalized along the inner nuclear membrane (Fig. 3.4c). These mononuclear exudates were disorganized, with constituent leukocytes loosely adhering to one another. Some macrophages were sequestering infected hepatocytes, and an occasional macrophage was seen to have phagocytosed portions of a macrogamont (Fig. 3.4d). These cellular profiles first occurred during degeneration of macrogamonts at 15-18 d p.i. By 18 d p.i. many macrophages were phagocytically active, and many contained prominent phagolysosomes that sequestered amylopectin granules (Fig. 3.4e). These cells also contained numerous primary lysosomes, which in some cells were aligning with a phagosome and fusing with it (Fig. 3.4e). By 40 d p.i.,
macrophages tightly opposed one another, and formed discrete aggregates. Most constituent cells contained numerous primary lysosomes and multiple, complex, heterogeneous phagolysosomes (Fig. 3.4f). Although melanin was never a predominant pigment in these parasite-induced MA’s, some lesions contained melanin-bearing cells by 40 d p.i. Individual melanin granules occurred discretely within the macrophage cytoplasm or as small groups bound within phagolysosomes (Fig. 3.4g). In semi-thin sections, these granules appeared golden brown to black and were thus easily distinguished from macrophage lysosomes and from the granules of heterophilic and eosinophilic granulocytes. By 70 d p.i., macrophages composing the MA’s contained multiple, large, heterogeneous phagolysosomes, many of which still contained amylopectin granules (Fig. 3.4h). Macrophages contained few primary lysosomes. Most MA’s were bordered by a thin capsule of reticular cells at 70 d p.i. (Fig. 3.4h). By 150 d p.i., many macrophages comprising MA’s no longer contained material that could be recognized as having a parasitic origin, whereas in some of these macrophages, amylopectin was still recognizable (Fig. 3.4i). Constituent macrophages at 150 d p.i. contained multiple complex residual bodies and large lipid droplets (Fig. 3.4i). Primary lysosomes were rare in these cells.

Eosinophilic Granulocyte

The eosinophilic granulocyte (EGC) was a large cell averaging about 12.2 μm long x 8.4 μm wide (N=50). Histologically, its pale cytoplasm contained numerous large intensely eosinophilic granules. The nucleus was small, oval to elongated, and eccentric, and this cell often exhibited an elongated profile in histological sections. Ultrastructurally, the EGC contained mitochondria and occasionally a prominent Golgi complex, but it rarely exhibited free ribosomes or RER. The cytoplasm generally was rich with small membrane-bound vesicles (Fig. 3.5a, b). The prominent cytoplasmic granules were heterogeneous
in electron density, and were bounded by a single membrane. They consisted of a dense granular matrix, bounded on one surface by a thin fibrillar, cord-like para-crystalline inclusion (Fig. 3.5b). This structure extended at opposite poles of the granule into the cytoplasm (Fig. 3.5c). It was most evident in granules whose contents appeared to be precipitating. Granules in degranulating cells were morphologically heterogeneous. Granule contents often precipitated on the inside surface of the granule membrane, and in some cells several granules had fused (Fig. 3.3a, 3.5b).

**Heterophilic Granulocyte**

The heterophil was similar in size to the mononuclear phagocyte, averaging about 8.6 μm long x 7.0 μm wide (N=50). This cell was generally rounded in profile with a small oval, eccentric nucleus. Ultrastructurally, this cell was readily distinguished from the EGC. The prominent cytoplasmic granules were variable in size but smaller than the EGC granule; each was bounded by a single membrane, and the contents were generally homogeneous and electron-dense (Fig. 3.5d). They did not contain the fibrillar inclusions observed in the EGC granule; however, a prominent electron-lucent halo occurred between the granule membrane and the electron-dense core (Fig. 3.5e). The cytoplasm contained elongated mitochondria and large numbers of heterogeneous small membrane-bound vesicles. Golgi complexes and RER were rarely observed in this cell. We observed no degranulation per se of the heterophil; however, the granules of some cells were quite heterogeneous, with contents appearing to condense or precipitate. Cytochemical reaction by the DAB method indicated that only heterophil granules were positive for peroxidase enzyme (Fig. 3.5f).

A third type of granulocytic leukocyte occurred in the early inflammatory exudate (12-20 d). This cell differed morphologically from the heterophil by having a prominent Golgi complex, greater RER content, and different granule morphology (Fig. 3.5g). Granules were variable in
their election density and often presented a fine speckled appearance. The prominent electron-lucent halo exhibited by heterophil granules was not observed. This cell could not be distinguished from heterophils and EGCs in histological sections.

**Small Mononuclear Cell (Lymphocyte)**

Histologically, these cells resembled lymphocytes, having an average size of 4.1 µm x 3.3 µm (N=50). They were intensely basophilic with a small dense nucleus surrounded by a scant rim of cytoplasm. Ultrastructurally, this cell had a heterochromatic nucleus that was irregular in outline, occasionally with a deep cleft. The scant cytoplasm was simple and contained few organelles (Fig. 3.5h). Several elongated mitochondria were generally present, some cells contained small vacuoles, and free ribosomes were abundant. Rough endoplasmic reticulum was rare in this cell, and a Golgi complex was not observed.

**Plasma Cell**

A leukocyte that morphologically resembled a plasma cell occurred in small numbers in the early inflammatory exudate (15-30 d p.i.). This cell exhibited a prominent dilated RER containing a flocculent precipitate (Fig. 3.5i). The nucleus was heterochromatic and often exhibited a prominent indentation or cleft. A prominent Golgi complex was often observed at the nuclear indentation. Other examples of this cell type are illustrated in Figs. 3.2b and 3.4e.

**Histochemistry**

Histochemical aspects of hepatic MA formation are outlined in Table 3.1. PAS-positive material was diffusely distributed within the hepatic parenchyma at 20 d p.i. Degenerating gamonts were intensely PAS-positive. By 25 d p.i., much of this PAS-positive material occurred in discrete focal aggregations (early developing MA’s). Staining of
these foci with the PAS technique was strong until 50-60 d p.i., after which time the intensity of staining decreased. Sudan black B positive material was initially distributed throughout the parenchyma (associated with inflammatory cells), but after 30 d p.i., it was restricted to the developing MA's. The intensity of staining and the amount of material that stained positively for Sudan black B increased slightly with time. Material identified as lipofuscin followed a similar trend. Discrete melanin granules first occurred in developing MA's 30 d p.i. and increased in abundance with time, although much variability existed in the amount of melanin within individual MA's. Deposition of reticulum fibers first occurred at the periphery of MA's at 30 d p.i. and was observed in association with the delicate capsule of reticular cells in all samples. The parasite-induced hepatic MA's were negative for ferric iron (hemosiderin) at all time periods sampled.

Ultrastructure of Splenic and Renal Macrophage Aggregates

Splenic MA's in Rivulus marmoratus consisted of mononuclear phagocytes replete with cytoplasmic inclusions (residual bodies) resembling complex siderosomes (Fig. 3.6a). Some inclusions contained melanin granules; however, this pigment was not present in large quantities. MA's were bordered by a thin capsule of reticular cells joined to one another by desmosomes. Some mononuclear phagocytes located at the periphery of splenic MA's were often less mature than centrally located ones; they contained numerous primary lysosomes and phagolysosomes (Fig. 3.6b).

The ultrastructure of renal MA's was similar to that of splenic MA's except that residual bodies in the kidney macrophages exhibited greater morphological variability (Fig. 3.6c). Melanin, although present, was not a predominant pigment. Most cytoplasmic inclusions in renal macrophages resembled ceroid pigment, although some inclusions resembled siderosomes. As in the hepatic and splenic MA's, some of the
constituent macrophages located at the periphery of renal MA's were often less mature than the centrally located cells. They contained numerous primary lysosomes and prominent phagolysosomes, features of a phagocytically active cell in the process of digesting endocytosed material (Fig. 3.6d).

DISCUSSION

This study suggests that the formation of hepatic macrophage aggregates in *Rivulus marmoratus* represents an attempt by the host to sequester and degrade a specific stage of an intracellular protozoan parasite. Correlation between this ultrastructural analysis and a previous high-resolution light microscopic study (Vogelbein et al. 1987) indicates that a mononuclear phagocyte, probably derived from peripheral circulation, plays a prominent role in the morphogenesis of these structures. However, this hypothesis rests on the assumption that images taken at various times p.i. represent sequential alterations in the cells participating in MA formation. Several observations support this assumption: 1) development of the parasite was synchronous, and the inflammatory process was similar in all five specimens examined at each of the sampling times, 2) cell populations of developing and fully formed centers remained relatively stable, i.e. mitoses were rarely observed, 3) cytomorphological changes in the constituent mononuclear phagocytes evolved slowly, with far less variation occurring within samples than between them, and 4) morphological characterization at each stage was based on the most "mature" cells present; atypical cells always represented earlier stages of development.

We interpret the parasite-induced, hepatic MA's in *R. marmoratus* to represent the end stage of a chronic inflammatory process in which a mononuclear phagocyte plays a prominent role. As such, these structures
may be compared with granulomatous inflammatory lesions of the higher vertebrates. Adams (1976) defined the granuloma of homeotherms as a compact, organized aggregate of mature mononuclear phagocytes (macrophages, epithelial cells, and multinucleate giant cells), which may or may not be accompanied by accessory features such as necrosis or infiltration by other leukocytic cells. This definition acknowledges morphologic criteria only, and it allows distinction as a granuloma on histologic grounds alone. Functionally, the granuloma represents a nonspecific host response to substances that are highly resistant to degradation. Constituent macrophages phagocytose, denature, and metabolize the foreign material (Cheville 1983). In addition to their phagocytic and degradative abilities, macrophages also display prominent secretory functions and play a pivotal role in initiating humoral and cell-mediated immune responses (Steinman & Cohn 1974). In homeotherms, these cells arise in the bone marrow from a pool of rapidly dividing stem cells. They subsequently enter and circulate briefly in the bloodstream as monocytes, and ultimately migrate to sites of inflammation (Spector 1974). Within the tissues, these cells can mature further to become macrophages, epithelioid cells, and multinucleate giant cells (Adams 1974, 1975). Together this group of cells comprises the mononuclear phagocyte system of higher vertebrates (Van Furth et al. 1972).

Granulomatous inflammation has also long been recognized in fishes. It can be elicited by various etiologic agents including inert compounds such as talc (Balouet & Baudin Laurencin 1986) and carageenin (Timur et al. 1977), immunogenic substances including Bacillus Calmette Guerin (BCG) (Balouet & Baudin Laurencin 1986) and Freund's complete adjuvant (FCA) (Secombes et al. 1985), and various infectious agents including acid-fast bacteria (Timur et al. 1977, Leibovitz 1980, Majeed et al. 1981), Corynebacteria (see Wood & Yasutake 1956a), fungi (Agius 1978, McVicar & McLay 1985), tissue-dwelling sporozoans (Ferguson &
Roberts 1975, Lom & Dyková 1981, Dyková & Lom 1981, Vogelbein et al. 1987), and metazoan parasites (Finn 1970, pers. obs.). Granulomatous nodules also develop as a consequence of altered tyrosine metabolism (Tixerant et al. 1984) and following experimental induction of autoimmunity (Secombes et al. 1985). Specific functional aspects of piscine macrophages that have been studied include chemotaxis (Weeks et al. 1988), phagocytosis (Sakai 1984, Honda et al. 1985, MacArthur & Fletcher 1985), and microbicidal mechanisms (Chung & Secombes 1987, 1988, Secombes et al. 1988, Graham et al. 1988). These studies suggest that the biology of piscine macrophages, although less understood than that of homeotherm macrophages, is similar in many respects.

A feature that distinguishes the piscine MA from the classic granulomatous lesion is the abundance of sequestered pigments in the former. At least three different pigments occur in MA's. Histochemical analyses (Wood & Yasutake 1956b, Tokumaru & Ferri 1970, Roberts 1975, Agius 1979, 1980, 1981a, Brown & George 1985, Wolke et al. 1985, Blazer et al. 1987) and ultrastructural studies (Roberts 1975, Agius & Agbede 1984, Fulop & McMillan 1984) suggest that these pigments are melanin, hemosiderin, and the lipogenic substances ceroid or lipofuscin.

Melanin, a complex polymer derived from the oxidation of the amino acid tyrosine, is synthesized by specialized cells called melanocytes and melanophores (Riley 1980). This pigment forms within compact, electron-opaque granules called melanosomes. The macrophages of mammals and fishes are thought to acquire melanin by phagocytosing the dendritic processes of melanogenic cells or their extruded melanosomes (Agius 1985, Wolke et al. 1985, Ghadially 1988). The function of melanin in macrophages is speculative (Agius 1985). Hemosiderin, a hemoglobin-derived storage form of iron, forms within macrophages following phagocytosis and degradation of effete erythrocytes (Fulop & McMillan 1984, Agius 1985). Ferric iron, in the form of ferritin, is bound to transferrin within macrophages and either stored or mobilized to sites
of erythropoiesis, where it can be re-utilized. The distribution of these pigments is variable. Splenic aggregates generally contain significant amounts of hemosiderin, whereas the hepatic and renal aggregates normally do not (Agius 1979, 1981a, 1983, Agius & Roberts 1981). In contrast, macrophages in the kidney of certain primitive teleosts such as the salmonids contain significantly more melanin than corresponding cells in the liver and spleen (Agius 1980). However, electron microscopy has revealed that most of the melanin bearing cells in the trout kidney are melanocytes (Agius & Agbede 1984).

The predominant pigments sequestered by piscine MA’s are ceroid and lipofuscin (Agius 1985). In homeotherms, these lipogenic substances are thought to result from the oxidation of polyunsaturated lipids (Hartroft & Porta 1965) and this oxidation has been suggested to occur in fishes as well (Agius 1985, Agius & Agbede 1984). Both terms have been used to denote pigment granules that are yellow to brown in hematoxylin and eosin-stained tissue sections, PAS-positive, acid-fast, sudanophilic, and insoluble in various organic solvents. However, some investigators make distinctions between ceroid and lipofuscin based on their etiology. Lipofuscin accumulates in a variety of vertebrate tissues, particularly in the brain and heart, as a result of the normal aging process. It consequently has been called an "aging" or a "wear and tear" pigment (Tsuchida et al. 1987). In contrast, the term "ceroid" has been used to define those lipogenic pigments that occur in various animal tissues undergoing certain pathological conditions (Hartroft & Porta 1965). Its presence in abnormal amounts characterizes the clinical syndrome ceroid-lipofuscinosis (Batten’s Disease) in humans (Goebel et al. 1979) and similar conditions in various other mammals (Jolly et al. 1980). This pigment has been extensively studied in rats with dietary injuries induced by vitamin E-deficient diets and by intravenous injections of different fat emulsions (for review, see Hartroft & Porta 1965). Kajihara et al. (1975) distinguished between
ceroid and lipofuscin differently. They defined the pigments observed in parenchymal, neuronal, and muscle cells as lipofuscin. Those pigments are formed by autophagocytosis, with subsequent peroxidation of membrane lipids. Ceroid was distinguished by originating in macrophages following heterophagocytosis of unsaturated lipids with subsequent oxidation and polymerization. Other authorities (Tsuchida et al. 1987, Ghadially 1988) considered lipofuscin and ceroid to be similar substances, and have attributed morphological and histochemical variations in these pigments to differences in their stage of maturation.

The origin of ceroid and lipofuscin pigments in mammals has been controversial. Several authors have suggested a mitochondrial derivation in both mammals (for review, see Tsuchida et al. 1987); however, this hypothesis is not universally accepted (Brizzee & Ordy 1981). The presence of lysosomal enzymes in lipofuscin granules and ultrastructural similarities between these granules and lysosomes suggests that these pigments are lysosomally derived (Kajihara et al. 1975, Goebel et al. 1979, Tsuchida et al. 1987). Most authorities presently regard both ceroid and lipofuscin granules as residual bodies that contain undigested residues following auto- or heterophagocytosis, both in fishes and mammals (Agius & Agbede 1984, Agius & Couchman 1986, Tsuchida et al. 1987). Our studies support this hypothesis. Although hepatic MA's in R. marmoratus contain some melanin, most of the sequestered pigment appears to be lipid-derived. Ultrastructurally, these pigment granules are heterogeneous in size, shape, and composition. In the later samples (50-150 d), this material resembles the ceroid pigments previously described in fishes (Roald et al. 1981, Agius & Agbede 1984) and mammals (Hartroft & Porta 1965). Our morphogenetic analysis suggested that this pigment was derived from the intracellular degradation of C. funduli gamonts and host tissue debris by mononuclear phagocytes. We were able to document the sequential
alterations in macrophages with confidence because of their apparent inability to degrade amylopectin granules. These parasite-specific, ultrastructurally distinct granules effectively marked those cells that had phagocytosed parasite material, allowing us to trace the development of MA's temporally. Amylopectin initially occurred in free macrophages, later within macrophages forming loose focal aggregations, and ultimately only within the cells comprising mature MA's.

Likewise, the temporal changes identified in our histochemical analysis indicate that parasite and host tissue debris was degraded and chemically altered by macrophages. Intensely PAS-positive parasitic stages (gamonts) initially distributed randomly within the hepatic parenchyma, were sequestered by aggregating macrophages and became less PAS-positive over time. Concurrently, this parasite and host tissue debris exhibited an increased sudanophilia and acid-fastness, presumably as lipid peroxidation progressed. Solangi and Overstreet (1980) made similar observations in the natural definitive host, *Fundulus grandis*, although pigment formation in that species was less consistent and more transient. Presumably MA's formed in *R. marmoratus* because parasite development was largely arrested during gamogony in this aberrant fish host (Vogelbein et al. 1987). Large numbers of degenerating parasites and host tissue degeneration stimulated a vigorous chronic inflammatory response. This mixed cellular response initiated the formation of hepatic MA's in this fish host. Hawkins et al. (1981) observed a transient inflammatory response in *F. grandis* and *F. similis* experimentally infected with *C. funduli*. Cell types comprising the initial host response in those killifishes were similar to what was observed in this study; however, the outcome of the cellular response differed in the two studies. Whereas, the inflammatory infiltrates gave rise to MA's in *R. marmoratus*, inflammation in the natural definitive hosts (*Fundulus* spp.) subsided during sporogony with subsequent fibrotic encapsulation of the fully developed parasites. Some degeneration of
macrogamonts occurred in *F. grandis* and *F. similis* (see Hawkins et al. 1981); however, most of the parasites seemed to develop normally. Most likely, the degree of parasite degeneration accounts at least partially for the differences in the outcome of the host response to this infection.

Several studies indicate that MA's modulate immune functions in fishes. The early observation in *Tautogolabrus adspersus* by Mackmull & Michels (1932) that intraperitoneally injected inert materials such as colloidal carbon are ultimately transported to and sequestered by MA's has been verified in other species including the plaice, *Pleuronectes platessa*, by Ellis et al. (1976); goldfish, *Carassius aurata*, by Mori (1980) and Herraez & Zapata (1986); rainbow trout, *Onchorhynchus mykiss*, by Tatner & Manning (1985); carp, *Cyprinus carpio*, and rosie barb, *Barbus conchonius*, by Lamers & Parmentier (1985); and river lamprey, *Lampetra fluviatilus*, by Page & Rowley (1984). Particulate antigens such as sheep red blood cells (RBCs) and various bacterins (Secombes & Manning 1980, Lamers & Pilarczyk 1982, Maas & Bootsma 1982, MacArthur et al. 1983, Lamers & De Haas 1985, Lamers 1986, Herraez & Zapata 1986, 1987), and soluble antigens (Ag) such as bovine serum albumen (BSA) and human gamma globulin (HGG) (Ellis 1980, Secombes & Manning 1980) are also sequestered by splenic and renal MA's. Other evidence was provided by Ellis & de Sousa (1974), who demonstrated that radiolabeled lymphocytes migrate through MA's, and by Roberts (1975) who reported the occurrence of a prominent capsule of lymphoid cells associated with MA's in certain species.

Lamers and DeHaas (1985) observed that Ag processing in carp was similar to the two-phased process observed in mammals. *Aeromonas hydrophila* Ag injected intra-muscularly was initially immobilized and phagocytosed by macrophages of the splenic ellipsoids and by solitary cells of the splenic pulp and renal hemopoietic tissues. This Ag was subsequently localized extracellularly on the surface of cells within
and adjacent to MA’s, where it was retained for up to 12 months. This phenomenon coincided with the onset of antibody production and the presence of immunoglobulin (Ig) on the outer surface of constituent macrophages (Lamers 1986) suggesting that Ag might be retained here for long-term storage as immune complexes. These observations have prompted several workers to suggest that MA’s might represent the primitive analogue of the mammalian germinal center (Ellis et al. 1976, Agius 1985, Lamers 1986). These investigators suggested that the sequestration of antigenic foreign materials by MA’s provides a micro-environment for the antigenic stimulation of immuno-competent lymphoid cells much as it occurs in avian and mammalian germinal centers. Furthermore, the long-term retention of Ag by these structures has been interpreted to imply a role in immunological memory. Naturally and experimentally infected F. grandis (see Solangi & Overstreet 1980) and experimentally infected R. marmoratus (pers. obs.) appear refractory to re-infection. These observations in conjunction with the observed presence of plasma-like cells in the early lesions of R. marmoratus (this study), support the view that MA’s may perform an immune function.

The terminology currently used to identify the pigment-bearing macrophages of fishes is controversial. In advanced teleosts, aggregates of these cells have been variously named; however, the name most frequently encountered in the scientific literature is the term "melanin-macrophage center." Initially coined by Roberts (1975), the latter name stresses the presence and functional importance of melanin in these structures. However, the amount of melanin apparently varies considerably among different species, different organ systems, and even among macrophages comprising individual MA’s (Agius 1980, 1985). In R. marmoratus, as in many other advanced teleosts, melanin is not the predominant pigment that it is in salmonids, such as the rainbow trout, Onchorhynchus mykiss (see Agius & Agbede 1984). This is true for the experimentally induced hepatic MA’s in R. marmoratus as well as splenic
and renal MA's not associated with the infection in this species. The function of melanin in piscine macrophages has not been rigorously studied, although several investigators have speculated about the potential functions of this pigment (Roberts 1975, Wolke et al. 1984, Agius 1985). Melanin is thought to act as a free radical scavenger, protecting the macrophage from these highly reactive substances produced during lipid peroxidation reactions and perhaps during inflammation (Agius 1985). Wolke et al. (1984) hypothesized that melanin may also serve a destructive function because of its ability to oxidize NADH with the subsequent production of hydrogen peroxide. This substance in conjunction with peroxidase and iodide is bacteriocidal (Cheville 1983). Whether piscine macrophages can utilize phagocytosed melanin in these capacities requires experimental verification. Some of these uncertainties probably account for the diversity of names that have been applied to these structures. We believe that regardless of location and pigment content, these structures are most accurately described by the general term "macrophage aggregate" introduced by Wolke et al. (1984). This term is descriptive without stressing any one of the pigments that can be sequestered by these ubiquitous structures.
Fig. 3.1: Morphogenesis of hepatic macrophage aggregates in *Rivulus marmoratus* infected with *Calyptospora funduli*. a) Extensive damage to hepatic parenchyma during merogony (8 d p.i.). Hepatocyte membranes are indistinct, nuclei (arrows) are pyknotic and cytoplasm is extensively vacuolated. MT - meront of *C. funduli*, MZ - merozoite. Bar = 10 µm. b) Acute inflammatory response following parasite-induced tissue damage (12 d p.i.). HV - hepatic vein, RBC - red blood cells, MP - monocyte, L - lymphocyte, H - heterophil, E - eosinophilic granulocyte. Bar = 10 µm. c) Extensive mixed inflammatory response (IC) during gamogony (15 d p.i.). Macrogamonts (MG) developing in hepatocytes (H) within a parasitophorous vacuole (PV). Bar = 10 µm. d) Sequestration and degradation of macrogamont (MG) by mononuclear phagocytes (MP) at 15 d p.i. H - hepatocyte, A - amylopectin granules. Bar = 10µm.
Fig. 3.2: Morphogenesis of hepatic macrophage aggregates in *Rivulus marmoratus* infected with *Calyptospora funduli*. a) Phagocytosis of parasite and host tissue debris by mononuclear phagocytes (MP) at 18 d p.i. Amylopectin granules (A) in a degenerating macrogamont (MG) and within phagolysosome of phagocytes. Bar = 10 μm. b) Border of early macrophage aggregate (MA) at 25 d p.i., composed of macrophages containing large, multiple phagolysosomes and degenerating macrogamonts (MG). IC - cuff of inflammatory cells at periphery of MA (arrow heads), PC - plasma-like cell, H - hepatocyte. Bar = 10 μm. c) Macrophage aggregate at 40 d p.i. MG - degenerating macrogamont, RB - residual bodies, M = melanin granules, A - amylopectin. Bar = 10 μm. d) Edge of macrophage aggregate (MA) at 40 d p.i., H - hepatocyte, MP - mononuclear phagocyte (monocyte), C - ceroid (residual body). Bar = 10 μm.
Fig. 3.3: Morphogenesis of hepatic macrophage aggregates in *Rivulus marmoratus* infected with *Calyptospora funduli*.  a) Border of macrophage aggregate (MA) at 60 d p.i. IC - inflammatory cells, H - hepatocyte. Bar = 10 µm. b) Macrophage aggregate at 60 d p.i., O - oocysts, SP - sporocyst within oocyst, MG - degenerating macrogamont, RB - residual bodies (ceroid). Bar = 4 µm. c) Macrophage aggregate (MA) at 100 d p.i. consisting of mononuclear phagocytes (MP) replete with multiple large residual bodies. Bar = 10 µm. d) Macrophage aggregate at 150 d p.i. bordered by capsule of reticular cells (RC), stored lipid droplets (L), and pockets of amylopectin granules (A). Bar = 10 µm.
Fig. 3.4: Sequential changes in the mononuclear phagocyte of *Rivulus marmoratus* during formation of hepatic macrophage aggregates.  

a) Monocyte with prominent lysosomal complex (L) at 12 d p.i. M - microtubules. Bar = 1 µm.  
b) Cytoplasm of monocyte exhibiting lysosomes (L) and prominent Golgi complex (GC). Bar = 1 µm.  
c) Macrophage within hepatic parenchyma at 18 d p.i. IS - intercellular space, L - lysosomes. Bar = 1 µm.  
d) Macrophage at 20 d p.i. internalizing degenerating macrogamont (MG). IS - intercellular space. Bar = 1 µm.  
e) Macrophage at 20 d p.i. with prominent phagolysosome (PL) containing amylopectin granules (A) and numerous primary lysosomes (L) aligning and fusing with phagolysosome. PC - plasma cell. Bar = 1 µm.  
f) Macrophage within macrophage aggregate (40 d p.i.). Note multiple phagolysosomes (PL) containing amylopectin (A) and abundant primary lysosomes (L). Bar = 1µm.  
g) Macrophage (same cell illustrated in Fig. 3.2c) containing melanin granules (M) free in cytoplasm and within phagolysosomes and amylopectin (A). Bar = 1 µm.  
h) Macrophage at border of macrophage aggregate (80 d p.i.) with large residual bodies (RB) containing amylopectin (A). RC - reticular cell. Bar = 1 µm.  
i) Macrophage at 100 d p.i. (see Fig. 3.3c) replete with heterogeneous residual bodies (RB) and lipid (L). RC - reticular cell at periphery of macrophage aggregate, D - desmosome between the reticular cell processes. Bar = 1 µm.
Fig. 3.5: Host leukocytes associated with hepatic macrophage aggregate formation in *Rivulus marmoratus*.  a) Eosinophilic granulocyte.  GC - Golgi complex.  Bar = 1 μm.  b) Granules in eosinophilic granulocyte.  Arrowhead-fibrillar band bordering edge of granule.  Bar = 0.5 μm.  c) Granule morphology of eosinophilic granulocyte.  Arrowhead-fibrillar band of granule extending into cell cytoplasm.  Bar = 0.5 μm.  d) Heterophilic granulocyte.  Bar = 1 μm.  e) Heterophil cytoplasm, Arrowhead - heterophil granule with electron-dense core and electron-lucent halo.  Bar = 0.5 μm.  f) Peroxidase activity of the heterophil (H).  Monocuclear phagocytes (MP) and eosinophilic granulocytes were negative for peroxidase activity.  Section was not stained with uranyl acetate and lead citrate.  Note dark reaction product in heterophil granules.  Bar = 1 μm.  g) Granulocyte with features different from heterophilic and eosinophilic granulocytes.  GC - Golgi complex.  Bar = 1 μm.  h) Lymphocyte.  Bar = 1 μm.  i) Plasma cell, GC - Golgi complex.  Bar = 1 μm.
Fig. 3.6: Splenic and renal macrophage aggregate ultrastructure in *Rivulus marmoratus*. a) Splenic macrophage aggregate (MA) consisting of macrophages replete with large residual bodies, some of which contain melanin granules (M). Bar = 10 µm. b) Higher magnification at edge of the aggregate illustrated in Fig. 3.6a (indicated by arrowhead). Mononuclear phagocyte (MP) at periphery of aggregate shows evidence of lysosomal activity, L - primary lysosomes, PL - phagolysosomes, RC - reticular cell at boundary of macrophage aggregate, M - melanin granule, RB - residual body resembling complex siderosome. Bar = 1 µm. c) Renal macrophage aggregate (MA) replete with heterogeneous residual bodies (RB). M - melanin, HT - hemopoietic tissue. Bar = 10 µm. d) Periphery of renal aggregate. The mononuclear phagocyte (MP) at the periphery shows evidence of lysosomal activity. PL - phagolysosome, L - primary lysosome, M - melanin, RB - residual body, RC - process of bordering reticular cell. Bar = 1 µm.
Table 3.1: Histochemical characteristics of hepatic macrophage aggregates in *Rivulus marmoratus* experimentally infected with the coccidium *Calyptrospora funduli*. + = weak positive reaction, ++ = moderate reaction, +++ = strong positive reaction, - = no reaction.

<table>
<thead>
<tr>
<th>Histochemical Stain</th>
<th>Days Post Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>AFIP for Lipofuscin</td>
<td>-</td>
</tr>
<tr>
<td>Sudan Black B (ceroid)</td>
<td>-</td>
</tr>
<tr>
<td>Schmorl’s (Melanin)</td>
<td>-</td>
</tr>
<tr>
<td>Perls’ (FerricIron)</td>
<td>-</td>
</tr>
<tr>
<td>PAS (carbohydrates)</td>
<td>-</td>
</tr>
<tr>
<td>Gomori’s (Reticulum)</td>
<td>-</td>
</tr>
</tbody>
</table>
LITERATURE CITED


Tatner, M. F., Manning, M. J. (1985). The ontogenetic development of the reticulo-endothelial system in the rainbow trout Salmo gairdneri Richardson. J. Fish Dis. 8:35-41


CHAPTER 4: THE EFFECTS OF CADMIUM CHLORIDE ON DEVELOPMENT OF EXPERIMENTALLY INDUCED HEPATIC MACROPHAGE AGGREGATES AND GRANULOMAS IN RIVULUS MARMORATUS

INTRODUCTION

Chronic exposure to sublethal concentrations of chemical toxicants can predispose aquatic organisms to disease (e.g. Zeeman and Brindley, 1981; Sindermann, 1988). However, the effects of environmental pollutants on specific host defense mechanisms in fish are poorly understood. Macrophage function is one aspect of cellular immunity that is reported to be adversely affected by exposure of fish to chemical contaminants. Weeks and Warinner (1984, 1986) and Warinner et al. (1988) observed greatly reduced phagocytic, chemotactic, and chemiluminescent responses in macrophages from fishes inhabiting a polycyclic aromatic hydrocarbon (PAH) contaminated environment. Rice and Weeks (1990) postulated that exposure to high concentrations of tributyltin caused membrane dysfunction, resulting in inhibition of calcium flux and depression of chemiluminescence in peritoneal macrophages of oyster toadfish (Opsanus tau). In contrast, exposure of carp (Cyprinus carpio) to the insecticides lindane and atrazine had no effect on in vitro phagocytic activity of macrophages (Cossarini-Dunier, 1987). Likewise, exposure of rainbow trout (Oncorhynchus mykiss) to 2,3,7,8-tetrachlorodibenzo-P-dioxin (TCDD) did not suppress phagocytic function of peritoneal macrophages, even at doses causing clinical toxicosis (Spitzbergen et al., 1986). These studies suggest that modulation of macrophage function in fish is toxicant-specific. Weeks et al. (1989) postulated that alterations in immune activity levels might be useful to characterize the health status of fish inhabiting chemically contaminated environments.

Pathological responses in aquatic organisms have also been suggested as indicators of environmental stress and fish health. For
example, an elevated prevalence of liver neoplasia has been linked with exposure of feral fish to chemical carcinogens in urbanized coastal environments (Myers et al., 1987; Baumann, 1989; Vogelbein et al., 1990). Similarly, macrophage aggregates (MA's) are often elevated in number and size in fish from degraded environments (Wolke et al., 1985a; Blazer et al., 1987; Bowser et al., 1990). These focal aggregations of macrophages sequester various pigments including ceroid, lipofuscin, hemosiderin, and melanin. MA's are considered to be a prominent component of the piscine reticulo-endothelial system (Roberts, 1975), and the sequestration of debris and pigments by the constituent cells may in some instances reflect their prior involvement in various pathological processes (Agius, 1985; Wolke et al., 1985b). MA's have been demonstrated to play a role in antigen processing and immunological memory (Lamers and De Haas, 1985; Lamers et al., 1987), and several investigators consider them to be a primitive analogue of the mammalian germinal center (Ellis et al., 1976; Ferguson, 1976; Agius, 1985; Lamers et al., 1987). Although MA's occur in many different tissues, they have been observed predominantly in the spleen, kidney, and liver. They apparently occur in all species of fish (Agius, 1980), require only routine histological methods for identification, and are readily quantified by morphometric methods. Consequently, they have been proposed as non-specific histological indicators of fish health and environmental quality (Wolke et al., 1985a; Blazer et al., 1987).

We recently reported that the intracellular coccidian parasite *Calyptospora funduli* elicits formation of hepatic MA's in the cyprinodontid teleost *Rivulus marmoratus* (Vogelbein et al., 1987). The ability to produce experimental infections has allowed us to define the morphogenesis of these structures and to clarify the association between the parasite and developing MA's. A detailed ultrastructural and histochemical study indicates that macrophages comprising the experimentally induced MA's are derived from blood monocytes that have
migrated into the hepatic parenchyma and subsequently phagocytosed degenerating parasites and host tissue debris (Vogelbein et al., submitted).

Our ability to elicit the induction and development of hepatic MA's with the Rivulus/Calypotospora model at specific times after infection presents an opportunity to test the potential effects of toxicant exposure on MA development under controlled laboratory conditions. The objective of this study is to quantitate potential effects of chronic cadmium chloride exposure on the development of experimentally induced hepatic MA's as well as granulomas in R. marmoratus, using morphometric analysis. We chose cadmium because numerous studies indicate that sub-lethal exposure to this heavy metal suppresses immune functions, both in mammals (Faith et al., 1980; Dean et al., 1982;) and in fishes (Robohm and Nitkowski, 1974; Zeeman and Brindley, 1981; Robohm, 1986).

MATERIALS AND METHODS

Experimental Infection of Fish

A total of 132 laboratory-reared adult (1 year old) specimens of R. marmoratus was used in this experiment. Sixty-six rivulus were experimentally infected with C. funduli according to the method developed by Fournie and Overstreet (1983). Briefly, about 150 adult feral grass shrimp (Palaemonetes pugio) were individually fed portions of liver tissue from naturally infected Gulf killifish (Fundulus grandis). Only livers heavily infected with sporulated oocysts (with infective sporozoite stage) of C. funduli were used to infect the shrimp. Grass shrimp were fed on three separate occasions over a 3-d period. Animals that did not feed were discarded. Following infection, shrimp were held for 7 d in two 55-l aquaria equipped with undergravel filtration. Water temperature and salinity were maintained at 25° C and 15 ppt, respectively. Shrimp were fed 24-hr old nauplii of Artemia
franciscana (previously *A. salina*) once a day and supplemented with Kordon stress flakes on every second day. After this holding period, 66 specimens of *rivulus* were infected with *C. funduli* by feeding them the freshly dissected hepatopancreas and alimentary tract of two infected grass shrimp. Feeding of infective shrimp tissue was conducted in small finger bowls so that individual fish could be observed. Individuals that did not consume both pieces of shrimp tissue were not used.

**Acute Mortality Test**

A 96-hr acute mortality test ($L_{C90}$) was conducted to permit estimation of an appropriate cadmium concentration for the chronic exposure test. Fifty uninfected adult rivulus, comprising five groups of 10 fish each, were exposed for 96 hr to 0.0, 10.0, 20.0, 40.0, and 60.0 mg/l nominal concentrations of cadmium chloride ($CdCl_2 \cdot 2.5H_2O$). A 96-hr $L_{C90}$ was calculated by Probit analysis using the ASTM Sample Probit computer program (Version 5).

**Protocol For Chronic Exposure**

Experimentally infected fish were randomly assigned to two treatment groups, each comprising 33 individuals. Uninfected fish from the same laboratory-reared stock were similarly assigned to two additional groups. Treatments groups were as follows: infected, 0 ppb Cd$^{++}$; infected, 250 ppb Cd$^{++}$; uninfected, 0 ppb Cd$^{++}$; and uninfected, 250 ppb Cd$^{+}$. Exposures were conducted in four 55-l glass aquaria. Prior to introduction of fish, we dosed the exposure system with exposure media to load glass and nytex surfaces with cadmium. Dosing was initiated 5 d prior to the start of the experiment. Solutions were renewed three times during this pre-exposure period. Infected fish were allowed to recover for 24 h following feeding of shrimp tissues before being transferred to the system. To eliminate aggressive interactions and assure continuous contact with water, fish were held individually in small nytex mesh baskets during the exposure period. Test solutions
were renewed every second day until day 34 of the experiment, after which they were renewed on every fourth day. Exposure of the fish was terminated on day 50 post-infection.

**Chemical Analysis**

Water samples from the acute mortality test and the chronic exposure study were analysed for total cadmium concentration using an Instrumentation Laboratories Model 351 atomic absorption spectrophotometer (AAS), equipped with a single element cadmium hollow cathode tube lamp (Jarrel-Ash), and operated at settings of 228.8 nm, with a 320 milli-μm slit, a 1-μm band path, and a current of 3 mA. A commercial cadmium standard (Cd AA StandARd [TM] Mallinckrodt) was checked by comparison with a standard prepared in the laboratory by dissolving 1.000 g metallic cadmium in a minimum of HCl and diluting to 1000 ml. The commercial standard was used for all subsequent analyses. For analyses, all standards and blanks were made up to 15 ppt using Ultra-pure¹ NaCl (Alpha Products) and water that was 1) glass-distilled, 2) cleaned in Water IR (Bronstead), and 3) redistilled in a fused quartz still.

**Tissue Preparation**

Fish were killed by overdose with tricaine methane-sulfonate. Livers and spleens were dissected from fish; livers were cut randomly into several pieces, followed by fixation of tissue blocks for 2 h in 1.25 % glutaraldehyde and 2.0 % paraformaldehyde in 0.1 M sodium cacodylate buffer (pH=7.4). Tissues were dehydrated in a graded ethanol series and then infiltrated and embedded in a glycol methacrylate embedding media (LKB Historesin®). Tissue blocks were sectioned at 2-3 μm on a Reichert Ultracut E³ ultra-microtome using glass knives. Tissue sections were mounted on glass slides and stained with Harris' hematoxylin and eosin-phloxine.

**Morphometric Analysis**

Quantitation of MA's, granulomas, and parasitic parameters was
performed on two groups of fish with an IBM Personal Computer equipped with Bioquant System IV® software and digitizer tablet. One group consisted of experimentally infected, exposed individuals and the other group of infected but unexposed fish. Randomly selected histologic sections were analysed using a light microscope with a camera lucida focused on the digitizer tablet. Tissue sections were randomized in the following manner. Livers were sliced with a single edge razor blade into five sections. The first slice was made randomly without regard to the orientation of the organ. The subsequent three slices were made parallel to the first cut. The five tissue slices generated in this manner were then embedded as one tissue block. Tissue blocks were faced until a full profile of each individual tissue slice was obtained. Hepatic parameters subjected to quantitation were 1) area of tissue section occupied by MA’s (% MA area), 2) area of tissue section occupied by granulomas (% GR area), 3) number of oocysts of *C. funduli* per mm² of tissue section (# OO/mm²), 4) number of MA’s per mm² of tissue section (# MA/mm²), and 5) number of granulomas per mm² (# GR/mm²). Splenic MA parameters measured were 1) area of tissue section occupied by MA’s (% MA area) and 2) number of MA’s per mm² of tissue section (# MA/mm²).

Treatment groups were compared for statistically significant differences using the Mann-Whitney U test. Probabilities ≤ 0.05 were considered significant.

RESULTS

96-Hr *LC₉₀* Test: Data from the acute mortality test are illustrated in Table 4.1. A 96-hr *LC₉₀* of 20.47 ppm total Cd⁺⁺ was calculated for adult rivulus with the 95% fiducial limits of 15.39 and 26.19. That *LC₉₀* value was used to estimate an appropriate Cd⁺⁺ concentration for the chronic exposure study. We chose a nominal concentration of 250 ppb (approximately two orders of magnitude below the *LC₉₀* value) to minimize the risk of mortality caused by potential synergistic effects between
the experimental infection and concomitant toxicant exposure.

**Chemical Analyses:** Total measured Cd\(^{++}\) concentrations for the chronic exposure study (Fig. 4.1) fluctuated during the 50-d exposure period and tended to be lower than the chosen nominal concentration of 250 ppb. Fluctuations were greater during the latter half compared with the first half of the experiment. No relationship was evident between the observed fluctuations and the time of sampling (pre- or post-renewal). Cd\(^{++}\) concentrations in control tanks remained below detection limits of the AAS throughout the study.

**Morphometric Analysis:** Grossly visible hepatic lesions developed in all of the experimentally infected fish. Histologic comparison of the infected, exposed individuals with the infected, unexposed individuals revealed no obvious qualitative differences in the hepatic MA's and granulomas. MA's were present in the spleens of these fish; however, no recognizable parasitic material was observed in this organ. Moreover, granulomas did not occur in the spleen. The two groups of uninfected fish (exposed and unexposed) did not develop hepatic MA’s or granulomas and were not analysed further.

In contrast to the lack of qualitative differences, quantitative differences in hepatic MA, granuloma, and parasitic parameters, as well as splenic MA parameters occurred between infected, exposed fish and infected, unexposed fish (Table 4.2). Percent MA area and number of MA’s per mm\(^2\) in the liver both were significantly lower in the infected fish exposed to 250 ppb Cd\(^{++}\) than in the group of infected, unexposed fish, whereas percent of area occupied by granulomas was significantly higher in the infected, exposed fish. Although the number of granulomas per mm\(^2\) appeared slightly elevated in the exposed group, this difference was not statistically significant. The number of oocysts per mm\(^2\) was significantly lower in the fish exposed to Cd\(^{++}\). In the spleen, percent of area occupied by MA’s was not significantly different between the two treatment groups; however, the number of splenic MA’s per mm\(^2\) was
significantly lower in the exposed fish.

DISCUSSION

This study is an attempt to develop an experimental approach that will allow quantitative evaluation of the potential effects of individual chemical toxicants on macrophage function in vivo. The rationale for using the *Rivulus/Calyptrospora* model is that the hepatic MA’s and granulomas elicited with this system represent the outcome of a chronic inflammatory process in which the macrophage plays a prominent role (Vogelbein et al., 1987). These structures thus represent histologic manifestations of recent macrophage activity within the liver of experimentally infected *R. marmoratus* that can be quantified. MA’s and granulomas were not observed in the livers of uninfected fish.

This morphometric analysis suggests that the parasite-elicited hepatic MA’s and granulomas in *R. marmoratus* are sensitive to the effects of chronic CdCl₂ exposure. Histologically, the appearance of hepatic MA’s, granulomas, and parasites (oocysts) did not differ in Cd⁺⁺-exposed and -unexposed fish. However, quantitative differences in those three features suggest that exposure to 250 ppb Cd⁺⁺ adversely affected survival of the parasite. The significantly lower number of oocysts per mm² of tissue section in livers of exposed fish suggests that fewer parasites sporulated successfully in that group. Hepatic MA’s were smaller and fewer in number in exposed fish, suggesting that an adverse effect of Cd⁺⁺ on parasite development occurred early, probably during merogony. Consequently, cadmium exposure may have had an adverse effect on the production of merozoites and gamonts. A smaller number of degenerating parasites and a smaller amount of tissue damage could explain the lower MA values observed in the exposed group. Although the number of granulomas per mm² was not significantly different between groups, the relative area of the tissue section occupied by granulomas was significantly greater in the exposed fish.
This difference suggests that granulomas were larger in the exposed group in spite of the presence of significantly fewer oocysts, and that macrophage recruitment may have been augmented in the Cd\textsuperscript{++}-exposed fish. On the other hand, cadmium may also have exerted an adverse effect on the later developmental stages of the parasite. The increased size of granulomas in the exposed fish may thus reflect an augmented macrophage response to a larger number of degenerating oocysts, rather than a direct effect of this heavy metal on macrophage activity.

Advantages of an experimental approach using the *Rivulus/Calyptospora* model for MA analysis include 1) a detailed knowledge of the stimulus for MA and granuloma formation, 2) the ability to analyse MA's at different stages during their development, 3) a knowledge of the history of experimental animals (i.e. sex, age, and health history of laboratory stock), and 4) the ability to manipulate environmental parameters during the culture of experimental animals and during exposure studies. Most of this important information is generally not available in field studies evaluating the effects of chemical contaminants on MA's. However, a disadvantage of this system is that interpretation of results may be complicated by potential effects of the toxicant directly on the parasite, as suggested in this study.

Macrophage aggregates already have been proposed as potential histological indicators of fish health and environmental quality (Agius, 1985; Wolke et al., 1985a,b; Lloyd, 1988). However, studies of the association between these structures and environmental health appear to be contradictory. MA's are reported to be larger and more abundant in fish from certain chemically contaminated (Haensly et al., 1982; Wolke et al., 1985b; Langdon, 1986; Myers et al., 1987; Lloyd, 1988; Vogelbein et al., 1990, Bowser et al., 1990) and thermally polluted environments (Blazer et al., 1987). On the other hand, dab (*Limanda limanda*) from heavily polluted sites in the German Bight exhibited fewer splenic MA's
than fish from nearby less contaminated sites (Kranz, 1988). Similarly, winter flounder (*Pseudopleuronectes americanus*) exposed to crude oil exhibited a decline in the number of hepatic MA's with increasing oil concentrations (Payne and Fancey, 1989). Laboratory exposure of plaice (*Pleuronectes platessa*) to potassium dichromate caused a three-fold increase in the number of splenic MA's; however, a concomitant three-fold decrease in their size caused the percentage of the tissue section area occupied by MA's to remain the same (Kranz and Gercken, 1987). Cunner exposed to high concentrations of cadmium exhibited a reduction in the hemosiderin content of renal MA's (Newman and MacLean, 1974). Reductions in the number, size, or pigment content of MA's in these studies were attributed to supression of macrophage activity, presumably caused by exposure to the chemical toxicants. Clearly, responses by MA's to toxicant exposure can be variable. These structures apparently form in response to endogenous or exogenous particulate antigens and represent an attempt by the host to sequester and degrade this material. Quantitative changes observed in the MA's of fishes exposed to chemical contaminants may thus reflect 1) an elevation in toxicant-induced tissue damage with a subsequent increase in the aggregation of mononuclear phagocytes replete with phagocytosed cellular debris (causing an increase in the size and number of MA's), 2) a direct toxicant-mediated suppression of macrophage function (resulting in a decrease in MA size and number), or 3) an indirect effect of a chemical toxicant on MA's mediated through interacting factors such as infectious agents.

Cadmium is an environmental contaminant that originates from the battery, plastics, electro-plating, and petroleum industries. Like most other heavy metals, cadmium does not degrade in the environment (Eisler, 1985). The effects of this metal on immune function in laboratory rodents include altered host resistance to pathogen challenge and suppression of humoral and cell-mediated immunity (reviewed by Faith et al., 1980; Dean et al., 1982). However, both suppressive and
stimulatory effects on immune function have been reported. Intraperitoneal injection of B 10-A-2R mice with cadmium delayed formation and depressed the number of splenic IgG and IgM plaque-forming cells following injection of sheep red blood cells (SRBC) (Bozelka et al., 1978). Exposure to high concentrations of Cd^{++} depressed antibody responses in rats, whereas low concentrations augmented them (Jones et al., 1977). Similarly, peritoneal macrophages of mice chronically exposed to cadmium were shown to have enhanced phagocytic activity and elevated acid phosphatase levels (Koller and Roan, 1977). Cadmium chronically administered to CBA/H mice in the drinking water delayed clearance of FC-bearing particles from the blood stream (Vredevoe et al., 1985) and inhibited in vitro phagocytic activity of peritoneal macrophages (Levy et al., 1986).

The effects of cadmium on piscine immune functions have received little attention. Exposure of cunner (Tautogolabrus adspersus) to Cd^{++} caused an elevation of bacterial uptake by phagocytes of the liver and spleen; however, the rate of bacterial killing by these cells was significantly decreased (Robohm and Nitkowski, 1974). Antibody production against IP-injected SRBC was not affected by Cd^{++} exposure in this fish. In contrast, O'Neill (1981) reported a substantial reduction in serum antibody titers to MS2 bacteriophage in cadmium-exposed brown trout (Salmo trutta). Similarly, Robohm (1986) found that exposure of cunner to 12 ppm Cd^{++} depressed serum antibody titers to IP-injected, formalin-killed Bacillus cereus in Freund's complete adjuvant. However, antibody response in striped bass (Morone saxatilis) exposed to 10 ppm Cd^{++} was enhanced six-fold over unexposed controls (loc cit.). Thuvander (1989) examined the effects of chronic exposure to low concentrations of cadmium on several measures of immune function in rainbow trout. In trout exposed to 3.6 µg Cd /L for 9 weeks, the cellular response to Vibrio anguillarum was significantly lower than in unexposed fish. However, the humoral antibody response to V.
_anguillarum_ O-antigen was higher in the exposed fish. Differential leukocyte counts and phagocytosis were not affected by cadmium exposure. Results of those studies are difficult to evaluate and compare because of differences in doses, routes of administration, duration of exposure, and immune parameters evaluated. However, they do suggest that the effects of Cd^{2+} on piscine host defense mechanisms require further study, and that results obtained with one species cannot necessarily be extrapolated to another.
Figure 4.1: Measured aqueous cadmium concentrations (total) in water during exposure of Rivulus marmoratus infected with Calyptospora funduli. a) Aquaria containing uninfected controls. b) Aquaria containing experimentally infected fish. (---), pre-renewal sample, 250 ppb; (-----), post-renewal sample, 250 ppb; (----), undosed aquaria.
Table 4.1: Test concentrations and mortality data for *Rivulus marmoratus* exposed to CdCl$_2$·2.5H$_2$O in synthetic sea water (15 ppt, 24°C, pH = 8.4).

<table>
<thead>
<tr>
<th>CONCENTRATION (ppm)</th>
<th>MORTALITY (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hr</td>
</tr>
<tr>
<td>Nominal</td>
<td>Measured$^a$</td>
</tr>
<tr>
<td>0$^b$</td>
<td>0.0</td>
</tr>
<tr>
<td>10</td>
<td>13.3</td>
</tr>
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<td>20</td>
<td>27.5</td>
</tr>
<tr>
<td>40</td>
<td>46.7</td>
</tr>
<tr>
<td>60</td>
<td>68.6</td>
</tr>
</tbody>
</table>

$^a$Total [Cd$^{++}$] measured at start of test.

$^b$Ten fish per treatment.
Table 4.2: Morphometric comparison of macrophage aggregates (MA), granulomas (GR), and parasitic involvement (Oo) in cadmium-exposed (250 ppb) and unexposed *Rivulus marmoratus* experimentally infected with *Calyptospora funduli*.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>EXPOSED</th>
<th>UNEXPOSED</th>
<th>Z-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LIVER</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>% MA Area</td>
<td>6.86 ± 1.23</td>
<td>11.47 ± 1.51</td>
<td>2.07*</td>
</tr>
<tr>
<td># MA/mm²</td>
<td>5.08 ± 0.48</td>
<td>7.02 ± 0.46</td>
<td>3.22*</td>
</tr>
<tr>
<td>% GR Area</td>
<td>1.84 ± 0.36</td>
<td>0.98 ± 0.36</td>
<td>2.32*</td>
</tr>
<tr>
<td># GR/mm²</td>
<td>1.39 ± 0.25</td>
<td>1.00 ± 0.29</td>
<td>1.69</td>
</tr>
<tr>
<td># Oo/mm²</td>
<td>31.23 ± 7.41</td>
<td>53.46 ± 5.33</td>
<td>2.07*</td>
</tr>
<tr>
<td><strong>SPLNE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% MA Area</td>
<td>4.54 ± 0.66</td>
<td>5.91 ± 0.99</td>
<td>0.52</td>
</tr>
<tr>
<td># MA/mm²</td>
<td>19.94 ± 1.41</td>
<td>28.69 ± 2.37</td>
<td>2.59*</td>
</tr>
</tbody>
</table>

*Hepatic samples: exposed (n=31), unexposed (n=29).
Splenic samples: exposed (n=29), unexposed (n=28).
Values represent mean of original measurements ± SEM.
'Significant difference, Mann-Whitney U test: $Z \geq t_{o.a; n-1}$
REFERENCES CITED


CHAPTER 5: SUMMARY

The piscine coccidium *Calyptospora funduli* elicits the formation of hepatic macrophage aggregates (MA’s) and granulomas in the estuarine cyprinodontid teleost *Rivulus marmoratus*. The ability to experimentally infect rivulus with this intracellular parasite provided an opportunity to investigate for the first time the sequential development of hepatic MA’s and to evaluate the role that these structures play in an on-going infectious disease process. Furthermore, this model system permitted the morphological characterization of MA’s at various stages of their development including a histological, histochemical, and ultrastructural descriptive study of the host leukocytes that participate in MA formation, and a study of the morphogenesis of the predominant pigments that are sequestered by these structures. Additionally, the host-parasite system was employed as a model to examine the effects of the heavy metal cadmium chloride on macrophage function *in vivo*.

SEQUENTIAL HISTOLOGICAL STUDY OF MA AND GRANULOMA FORMATION

1) Parasite degeneration is elevated in *Rivulus marmoratus* infected with *Calyptospora funduli*, probably because this small estuarine cyprinodontid is not a natural definitive host of this coccidian parasite.

2) The cellular host response to *C. funduli* in *R. marmoratus* differs from that in natural definitive fish hosts.

3) Hepatic MA’s and granulomas form in response to degeneration of the protozoan and necrosis of hepatic parenchymal cells.

4) MA formation is initiated during gamogony in response to degenerating gamonts and probably cellular debris from hepatocytes killed by merozoites, whereas granulomas are elicited during sporogony and develop in response to the oocyst stage.

5) Hepatic MA’s and granulomas elicited by *C. funduli* thus represent
endstages of a chronic inflammatory process in which a mononuclear phagocyte, apparently derived from the peripheral circulation, plays a prominent role.

SEQUENTIAL HISTOCHEMICAL EVALUATION OF HEPATIC MA's

1) Histochemical staining of hepatic MA's for carbohydrates, lipofuscin, melanin, lipid peroxidation products (ceroid), and reticulum fluctuated during the development of the parasite.

2) PAS-positive material was diffusely distributed throughout the hepatic parenchyma at the early sampling times (15-25 d p.i.). The gamonts of C. funduli stained intensely PAS-positive. During the intermediate sampling times (25-40d p.i.), the intensely PAS-positive parasitic stages became focally localized within developing MA's. PAS staining of MA's declined during the late sampling periods (50-150 d p.i.).

3) Peroxidized lipids (ceroid) were initially diffusely distributed and weakly Sudan-black B-positive. During the intermediate sampling periods this material also became focally localized within developing MA's. The staining of MA's with Sudan black B was most intense during the late sampling periods.

4) Parasite-elicited hepatic MA's contained no ferric iron.

5) Starting at 30 days p.i., the staining of MA's for lipofuscin, melanin, and reticulum fibers increased progressively increased.

ULTRASTRUCTURAL CHARACTERIZATION OF MA's in RIVULUS MARMORATUS

1) The inflammatory infiltrate initiated during late merogonic and early gamogonic development of the parasite is composed of six ultrastructurally distinct leukocytes. These include: 1) a mononuclear phagocyte, 2) a small mononuclear cell (lymphocyte), 3) a plasma-like cell, 4) an eosinophilic granulocyte, 5) a heterophilic granulocyte, and 6) a granulocyte morphologically
distinct from the other two granulocytes.

2) The fate of the mononuclear phagocyte, a cell that comprised a prominent component of the cellular infiltrate, can be followed over time because of its apparent inability to degrade the phagocytosed parasite-derived amylopectin granules.

3) Most of the pigment granules sequestered by MA's are large, heterogeneous inclusions that are ultrastructurally identical to material that has been identified as ceroid in mammals and fishes.

4) Ceroid granules are residual bodies that are retained within macrophages after the intracellular digestion of phagocytosed host and parasite-derived material.

5) Melanin granules are not abundant in the experimentally induced MA’s and appear to have been phagocytosed by the constituent macrophages.

6) Parasite-elicited hepatic MA’s resemble splenic and renal MA’s. The ultrastructural morphology of constituent cells is essentially the same in MA’s from all three organs except that pigment granules in the different sites differ structurally because of the different materials being degraded.

TOXICOLOGY/MORPHOMETRY

1) Quantitative changes in MA and parasite parameters were induced by chronic CdCl₂-exposure of experimentally infected fish.

2) Exposure to 0.25 ppm cadmium for 50 days caused a reduction in the number of oocysts that developed, the number of hepatic MA’s that formed, and the relative area of tissue sections occupied by the hepatic MA’s. The relative area in tissue sections occupied by granulomas was greater in exposed fish, whereas the number of granulomas was not. Splenic MA’s in exposed fish were greater in number, but the relative area of the section occupied was not significantly different.
3) Exposure to cadmium interfered with the development of *C. funduli*. Adverse effects of Cd-exposure on macrophage function were not demonstrated. Results support the hypothesis that MA's in the liver of *R. marmoratus* experimentally infected with *C. funduli* form in response to the infection. Results also suggest that effects of chemical toxicants on the parasite can be separated from potential effects of exposure on macrophage function. This host-parasite system may therefore be a sensitive model for further investigations on the adverse effects of toxicant exposure on *in vivo* macrophage activity.
Wolfgang Klaus Vogelbein was born in Bremen, West Germany on July 11, 1953. He emigrated to the United States in 1963 and subsequently grew up on Long Island, New York. He graduated with a Bachelor of Science degree in Marine Biology from Long Island University's, Southampton College in 1976. He received a Master's of Science degree in Zoology from California State University, Long Beach in 1981. His thesis for the Master's Degree was entitled "Parasites as Stock Indicators in Three Species of Antarctic Whales". His Program of Study at the California State University was directed by Dr. Murray D. Dailey. In 1983, he began graduate work in the Department of Veterinary Microbiology and Parasitology at the School of Veterinary Medicine, Louisiana State University. He established a joint Program of Study between LSU and the Gulf Coast Research Laboratory in Ocean Springs, Mississippi. Drs. Ronald L. Thune of LSU and Robin M. Overstreet of Gulf Coast Research Laboratory serve as his co-major advisors. He spent 1.5 years in residence at LSU to complete his course work and then went to Gulf Coast Research Laboratory to conduct his dissertation research. In 1989 He accepted a faculty position with the College of William and Mary's Virginia Institute of Marine Science in Gloucester Point, Virginia. He is in charge of the Electron Microscopy and Histopathology laboratories and is conducting research in Aquatic Animal Pathology. His research focuses on the association between environmental pollution and fish health. He is presently a candidate for the Doctoral degree in Veterinary Medical Sciences at Louisiana State University and is majoring in Parasitology with a minor degree in Marine Science.
Candidate: Wolfgang K. Vogelbein

Major Field: Veterinary Medical Sciences

Title of Dissertation: The Morphology, Development, and Function of Experimentally Induced Macrophage Aggregates in Rivulus marmoratus.

Approved:

[Signatures]

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

April 16, 1991