IDENTIFICATION OF MAJOR HISTOCOMPATIBILITY COMPLEX HAPLOTYPES

IN GOLDFISH, CARASSIUS AURATUS

by

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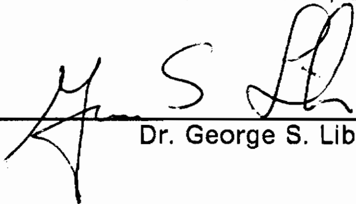

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Identification of Major Histocompatibility Complex Haplotypes

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Abstract

Development of techniques for observing variability at the major histocompatibility complex (MHC) of fishes could prove an important first step in understanding the genetic bases of disease resistance. In this study, using goldfish (*Carassius auratus*) as a model system, three approaches to generating antisera to putative MHC molecules and two methods for detecting antibody reactivities were evaluated.

Seven full-sib families were produced, and red blood cells (RBCs) of goldfish family members were screened for reactivity with a panel of absorbed antisera. The antisera panel consisted of fish anti-fish, chicken anti-chicken, and chicken anti-fish antisera. The fish anti-fish antisera was produced by injecting RBCs from each parent into its mate, and the chicken anti-fish antisera was produced by injecting parental goldfish RBCs into chickens. The chicken anti-chicken antisera were obtained from a genetics laboratory where MHC-specific antisera had been prepared previously. The pattern of presence or absence of agglutination upon mixing with the respective reagents in this panel of antisera was regarded as the phenotype of the individual tested.

Agglutinations observed macroscopically or microscopically were easily scored as positive or negative. Particular phenotypes were observed among individuals both within and between families.

The large numbers of phenotypes observed may indicate: (1) the need for additional absorptions in the preparation of antisera, or (2) segregation of additional sets of phenotypic MHC haplotypes in the tetraploid goldfish. The utility of chicken anti-chicken reagents in serotyping of fish was demonstrated.

Use of the traditional approach to conducting hemagglutination assays limited the number of assays executed because of the amount of blood required. In order to minimize the sample volumes required, antibody reactivities were evaluated by flow cytometry employing appropriate fluorescein labeled antibodies. Using this approach, scoring of positive and negative results was equivocal, and results did not always agree with those scored by hemagglutination assays.

Results of this study strongly suggest that the development of immune allo and xeno-antisera and use of hemagglutination assays can be used to characterize genetic variability of the MHC of fishes. Understanding of immunogenetic variability in fishes could be used to develop strains resistant to economically important fish pathogens.

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INTRODUCTION

The major histocompatibility complex (MHC) is a highly polymorphic gene complex that encodes glycoproteins expressed on the surface of nucleated cells of all vertebrate species studied. The MHC is an important component of the immune system, involved in cellular as well as humoral immunity. Variation in certain subregions of the MHC has been linked to variability in resistance or susceptibility to particular infectious agents. The MHC has been studied extensively in human and mouse. It has also been studied in chicken, pig, cattle, horse and other traditional agricultural species. However, the data and reagents necessary for MHC typing in aquaculture species are limited. Methodologies used to study the piscine MHC to date have limited practical utility. The major goal of this research was to develop a practical method for detecting MHC gene products in fish, so that the genetic bases of disease resistance could be better understood. Such knowledge could be applied in genetic improvement of disease resistance in aquaculture stocks.

THE MAJOR HISTOCOMPATIBILITY COMPLEX

Immunology is the study of the structure and function of the immune system. The function of the immune system is to distinguish self from non-self substances. Immunological competence includes three components, specificity, memory, and cytotoxic or antagonistic reaction following a latent period of sensitization (Hildemann 1977a).

The immune system is composed of two divisions: the humoral system, whose action is effected by antibodies, and the cellular system, whose action is effected by immune cells. In mammals, antibodies are produced by B lymphocyte-derived plasma cells in the lymph nodes and spleen. T lymphocytes, produced in the thymus, are the primary mediators of cellular immunity. In some species, the immune system depends on other types of cells (macrophages, eosinophils, basophils, neutrophils), proteins (complement, cytokines), and MHC in order to function properly. MHC gene products were discovered over 50 years ago, but their physiological function was not defined until 1974 (Zingernagel et al. 1974). MHC gene products are now collectively recognized as a major component of the immune system.

The MHC is a highly polymorphic gene complex, whose gene products are located on the surface of nucleated cells. Some immunologists define the MHC as a group of genes encoding for molecules that provide the context for the recognition of foreign antigens by T lymphocytes. Others argue that the complex could include genes that are not involved with the recognition of antigen by T cells (Klein 1986). These additional genes encode complement and other enzymes, and are physically located within the chromosomal segment occupied by the MHC.

MHC function has been demonstrated in mammals, birds, and amphibians. Indications of MHC function in Agnatha, higher fishes, and reptiles are based on observations of graft rejections, mixed lymphocyte reactions, and graft-versus-host reactions (Klein 1986). Except for the most primitive species, lymphocytes and the thymus gland, the origin of T cell production, have been found in all vertebrates. It is assumed that MHC-like molecules exist in all vertebrate species because of the presence of thymocytes (T cell precursors) and because of antibody responses demonstrated in graft rejection. However, immunogobulins have not yet been identified in some species which demonstrate graft rejection.

A PHYLOGENETIC OVERVIEW OF IMMUNE FUNCTION

A brief phylogenetic review of immune system structure and function will provide context for the problem chosen, MHC characterization in fishes. Incompatibility reactions have been observed in metazoan invertebrates ranging from coelenterates to protochordates (Hildemann 1977a). Self-incompatibility has been demonstrated in some protozoan species (Hildemann 1972a, 1978) and even in flowering plants (Burnet 1971, Klein 1986).

Self-incompatibility is common in **flowering plants** (Burnet 1971). Many flowering plants are hermaphroditic, a trait which can tend to reduce variation. Angiosperms have developed two incompatibility mechanisms which promote outbreeding (Klein 1986). According to the complementary mechanism hypothesis, pollen grains can germinate and produce short tubes in an incompatible style; alternatively, the incompatible pollen somehow fails to activate the nutrients, conditions, or stimuli necessary for growth. The oppositional mechanism hypothesis proposes that the growth of incompatible pollen tubes is actively inhibited by specific molecules.

Self-incompatibility systems in plants are not fully understood. There is considerable variation of genetic control and phenotypic expression among the different plant species. Some researchers feel that self-recognition in plants may be an early precursor to the MHC system. Others feel that the genes involved in this type of recogniton are not ancestral because the plant genes operate by carbohydrate-protein or carbohydrate-carbohydrate interactions, while MHC molecules are recognized by protein-protein interactions (Klein 1986).

Among the invertebrates, immunorecognition has been observed in many animals such as sponges, colonial hydroids, anthozoans (including sea anemones), corals, annelid worms, echinoderms, and tunicates (Hildemann 1977b, 1979).

Porifera (sponges) are the most primitive multicellular animals. Self-recognition in some species of sponges can be demonstrated by the reconstitution of whole animals from individual cell

suspensions. Individual cells allowed to settle in a dish of water will attach themselves to the substrate, adhere to one another on contact, and differentiate into functional sponges (Klein 1986). If two cell suspensions are prepared from different species of sponge and mixed together, the two cell types separately reconstitute to the two parental species, demonstrating that the cells are able to recognize one another (Hildemann 1972a, Hildemann et al. 1979, Klein 1986). An aggregation factor has been isolated which binds to a glycoprotein receptor on the cell surface. Aggregation factors are species-specific, and appear to be responsible for the failure of cells from different sponge species to aggregate (Klein 1986). Some species of sponges also have demonstrated acceptance of isografts and antagonistic rejection of allografts. These graft-versus-host reactions are suggestive of specific or selective immunity involving a memory component (Hildemann et al. 1979).

Among the **cnidarians** (coelenterates), evidence of allograft rejection has been demonstrated in Hydrozoa and Anthazoa. Anthazoans (sea anemones and corals) have been found to demonstrate allogeneic reactions (reactions between genetically dissimilar individuals within the same species) even though these animals lack a vascular system, organ systems, and lymphoid-type cells usually associated with vertebrate immune responsiveness (Hildemann et al. 1979). Examination of tissue grafts of scleractinian coral have revealed evidence of polymorphic histocompatibility markers, specificity, and at least short-term memory (Hildemann et al. 1977b).

Echinoderms constitute a major phylum which includes asteroids (sea stars), ophiuroids (brittle stars), echinoids (sea urchins), holothuroids (sea cucumbers), crinoids (sea lilies) and concentricycloids (sea daises) (Pearse et al. 1987). Some species of echinoderms have demonstrated immunorecognition involving rejection, specificity, and short-term immunological memory (Hildemann et al. 1972a, Karp and Hildemann 1976). These animals also exhibit the presence of leukocytes (coelomocytes), macrophages, hemocytes (lymphocytes), and eosinophils similar to those found in vertebrates (Hildemann and Dix 1972b). Skin allografts and autografts of sea cucumbers have been studied for immune responsiveness. First-set autografts remained intact for six months or longer,

while allografts began to show signs of rejection in 40-60 days. Areas of rejection were infiltrated with macrophages and hemocytes. Second-set allografts showed accelerated rejection times, demonstrating a short-term memory immunoresponse in these animals (Hildemann and Dix 1972b). Sea stars have demonstrated similar graft-versus-host reactions. With these animals, third and fourth-set allografts have demonstrated rapid rejection times of 4-13 days. These results suggest that echinoderms possess a well-developed, cell-mediated immune response analogous to that in vertebrates (Karp and Hildemann 1976).

The phylogenetic origin of the vertebrates is uncertain. However, the prevailing hypothesis is that vertebrates evolved from from sessile marine **urochordata** (invertebrate chordates) known as tunicates. These colonial animals exhibit a polymorphic genetic system controlling the compatibility of their colonies (Klein 1986). When two fragments derived from the same colony are placed next to each other (at their growing edges), they fuse to form a single colony. If fragments of two different colonies are placed together, one of two events may occur. In some species, fragments fail to fuse and distinct boundaries can be observed. In other species, necrosis occurs at the contact zone, followed by rejection of the fragment (Klein 1986). Scofield et al. (1982) determined that the nonfusion reaction is controlled by either a single heterozygous locus or a complex of loci with many codominantly expressed alleles. Fusion occurs between colonies that share at least one allele at the fusibility locus. The fusibility locus shows the same degree of polymorphism as the genes of the vertebrate MHC and the self-incompatibility systems of plants (Scofield et al. 1982). However, there are differences between this system and the vertebrate MHC. Fusion is allowed between colonies sharing only one allele. In vertebrate systems, lymphocytes destroy cells carrying non-histocompatible antigens. Also, it is not known whether allorecognition in tunicates is carried out by all cells, or only by ampullar and blood cells (Scofield et al. 1982).

A major immunological distinction between vertebrates and invertebrates is the ability to produce and secrete serum antibodies. Immunologic memory has been demonstrated in all classes

of vertebrates. However, the MHC has been identified in only 20 vertebrate species, including only one avian species and one species of amphibian (Klein 1986). Indirect evidence (graft rejection) indicates that the MHC may be present in fish and reptiles. These classes of vertebrates also have demonstrated the capacity to produce antibodies of at least the IgM type.

Because most studies of immunocompetence have been carried out with mammals, comparisons among broader phylogenetic taxa should consider variables such as response specificity, development and duration of immunologic memory, spectrum of cellular or humoral responses, immunogenetic library, and molecular classes and quality of antibodies produced (Hildemann 1970, 1972a). The types of antibodies produced in a given vertebrate species are a function of phylogenetic class. Some mammals produce an array of antibodies (IgM, IgG, IgE, IgD, IgA), while fish are known only to produce IgM (Warr and Marchalonis 1976, Ourth 1982, Secombes and Manning 1990).

The Piscine Immune System

Hagfish and lampreys, class Agnatha, are the most primitive living vertebrates. These fish apparently evolved from the first chordates, the ostracoderm Devonian fishes. Despite their primitive status, Pacific hagfish (*Eptratretus stoutii*) have a fully developed capacity to recognize and reject skin allografts (Hildemann 1970, 1972a). Temperature-dependent primary and secondary allografts have demonstrated specific immunity (involving lymphocyte infiltration), as well as persistent immunological memory (Hildemann 1970). The chronic nature of allograft rejection has been demonstrated in hagfish, which may indicate the presence of weaker histocompatibility mechanisms than those found in higher vertebrate species (Hildemann 1970). Rejection of primary allografts requires an average of 72 days, and a rejection of a secondary allograft, about 28 days. These primitive fishes produce serum antibodies which appear to be similar to IgM found in mammals.

Chronic rejection of initial skin allografts also appears to be characteristic of the sharks and rays (Chondricthyes). Primary allografts are rejected in an average of 41 days and secondary allografts are rejected in about 18 days (Hildemann 1972a). The chronic nature of allograft reactions is not the result of a deficiency in the immune response. Antibody titers do not appear to be lower in animals demonstrating chronic graft rejections. Weak primary incompatibilities can be strengthened by preimmunization with a specific antigen (Hildemann 1970).

The bony fishes, class Osteichthyes, exhibit increases in both transplantation immunity and serum antibody responses relative to more primitive fish classes (Hildemann 1972a). There are substantial data concerning the genetics of tissue transplantation in teleosts. The first investigations of histocompatibility genetics in fish involved cross-grafting of scales of adult goldfish (Hildemann 1972a). These studies showed allograft rejection to be as vigorous and as acute as reactions demonstrated in some mammalian species (Hildemann 1970). Goldfish demonstrate primary rejection in about 9 days, and secondary rejections occur in about 7 days. Studies using scale and fin allografts in platyfish and killifish have produced similar results (Kallman 1970).

Lymphocyte surface immunogobulins have been studied in goldfish *Carassius auratus* (Warr et al. 1976), brown trout *Salmo trutta L.* (O'Neill 1978), rainbow trout *Onchorynchus mykiss* (Etlinger et al. 1977), and carp *Cyrinus carpio L.* (Secombes et al. 1980, 1983; Kaastrup et al. 1989, Stet et al. 1990) in attempts to demonstrate immune responsiveness and the presence of MHC gene products. Studies suggest that lymphocytes of the thymus and spleen in these species express surface immunoglobulins. The IgM antibodies of goldfish are comprised of light and heavy chains identical to serum IgM of mammals (Warr et al. 1976). Goldfish also appear to express surface antigens capable of performing functions attributed to mammalian T cells and B lymphocytes. MHC molecules are expressed on the surface of goldfish lymphocytes and possibly also on erythrocytes (Stet et al. 1990). The presence of MHC class I and class II molecules is implied due to allograft rejection, mixed lymphocyte reactivity, and the requirement for antigen processing and presentation in antibody

reponses to thymus dependent antigens. More recently MHC class I and class II molecules have been described at the molecular level (Dr. Norman Miller, University of Mississippi Medical Center, pers. comm.).

The Amphibian Immune System

It is believed that amphibians emerged approximately 300 million years ago. Study of these lower vertebrates is useful in reconstructing the evolutionary history of the MHC, and may give insight into the functional relationships among its constituent loci (Du Pasquier et al. 1975). Acute alloimmune responses have been demonstrated in both larval and adult bullfrogs (*Rana catesbeiana*) (Hildemann 1972a). Chronic allograft rejection has been demonstrated in newts and salamanders (Hildemann 1972a).

In only one species, the South African frog (*Xenopus laevis*), has a genetic region that can be considered homologous to the MHC of other species been described (Du Pasqiuer et al. 1975, Bernard et al. 1979). This XLA (Xenopus lymphocyte antigens) region has been characterized by mixed leukocyte reaction (MLR), acute graft rejection, and by the presence of certain antigens on erythrocytes and lymphocytes. These data reveal two main homologies with higher vertebrates. When *Xenopus* hybrids were studied for MLR, it was determined that one locus segregates in these families, together with the genes controlling acute graft rejection. Furthermore, cytotoxic killer T cells have been isolated which are responsible for cell-mediated lympholysis (CML) as in mammals (Bernard et al. 1979). *Xenopus* cytotoxic cells, however, do not behave exactly like the mammalian counterpart; CML can only be generated after preimmunization with cells or skin grafts. In mammals, re-stimulation is not required for MHC reactivity, but is needed for generation of killer cells against minor histocompatibility antigens (Bevan 1977). These observations suggest that the *Xenopus* MHC may be functionally similar to minor histocompatibility loci of mammals (Du Pasquier et al. 1975). Despite these differences, it appears that the genes responsible for the highly specialized function of

killer T cells evolved at least at the time of the emergence of amphibians, and that these genes were already linked to the MHC (Bernard et al. 1979).

The Reptilian Immune System

Data concerning transplantation in reptiles are either preliminary or lacking in detail. However, histocompatibility antigens and transplantation immunity have been studied in the three major orders of reptiles (Chelonia, Squamata, and Crocodilia). The species studied (snapping turtle, chameleon, iguana, garter snake, caiman) have shown chronic rejection of primary allografts over times ranging from 21-245 days (Hildemann 1972a). Studies of reptilian blood have revealed that some species, such as turtles and alligators, express granulocytes (except perhaps neutrophils), lymphocytes, and plasma cells. The Florida alligator, representative of the most advanced order of crocodilian reptiles, produces IgM and IgG antibodies very similar to those previously reported only in birds and mammals. Extensive polymorphism of cellular alloantigens (including erythrocyte alloantigens) and histocompatibility antigens appears to be typical of many reptilian species (Hildemann 1972a).

The Avian Immune System

Definitive identification of MHC in birds has been reported in only one species, the chicken *Gallus gallus domesticus*. Gene products of the chromosomal region presently known to contain the MHC of chicken were first detected using alloimmune hemagglutinating reagents against chicken red blood cell antigens (Briles et al. 1948). Blood cells of other avian species (pigeon, ducks, turkeys, doves) have been screened for agglutination against specific antigens but none have been studied as extensively as the chicken (Briles and Briles 1982).

The MHC complex in chicken, referred to as the B locus, simultaneously functions as a blood group encoding locus and as a histocompatibility locus (Hildemann 1972a). The B system shows

extensive genetic polymorphism and controls alloreactivity, skin graft rejection, and graft-versus-host reaction. The involvement of MHC in cellular interactions, known as MHC restriction, has been demonstrated in the B system through mixed lymphocyte reactions, T cell stimulation by antigen presenting cells, T and B cell cooperation in antibody production, generation of germinal centers in the spleen, and stimulation of cytotoxic T cells (Guillemot 1989).

The B system is currently modelled a three-locus system, with the B-F and B-L loci demonstrating the biochemical characteristics of mammalian MHC class I and class II antigens, respectively (Guillemot 1989). B-F encoded molecules are expressed on both leukocytes and erythrocytes, while B-L encoded molecules are found only on erythrocytes. The third locus (B-G) is considered to be a recombinant, with strong linkage disequilibrium between B-F and B-G alleles. B-G does not appear to have a mammalian counterpart (Simonsen et al. 1981).

Study of large populations of outbred chickens has led to a collection of useful data that would have been difficult to generate using mammalian models. Such study has provided the best examples of the strong influence of the MHC on resistance to infectious disease. Briles et al. (1977) discovered that certain B haplotypes are more resistant to the herpes virus which induces Marek's disease in poultry. Nonimmune traits also have been associated with certain haplotypes in the B system, including egg production, hatchability, and weight of inbred chicks (Briles 1960).

The Mammalian Immune System

Most studies in immunology and immunogenetics have involved mammalian species. The MHC has been identified in mice (H-2), rats (RT1), sheep (OLA), cattle (BoLA), horses (ELA), pigs (SLA), dogs (DLA), humans (HLA), and in many of the primates (Klein 1986). The MHC is of interest to researchers studying domestic animals because these genes control disease resistance and susceptibility, and have been associated with reproductive success and fitness.

The H-2 system in mice was the first MHC system to be identified. It was discovered through graft-versus-host reactions and later through mixed lymphocyte reactions. Wild mice, as well as inbred strains, have been used in mapping the H-2 locus, which has provided information for identifying the MHC in other species. Development of DNA probes has provided a more specific method for characterizing the MHC, and some mouse probes cross-hybridize with other species (Klein 1986, Lew et al. 1986, Vaiman and Chardon 1986a).

H-2 loci are located on chromosome 17, and are divided into three classes (I, II, and III). Class I molecules are highly polymorphic and are involved in graft rejection, MHC restriction of T cell reactions, antibody production, and cell-mediated lysis reactions. Class II loci are involved in recognition of antigens presented by B cells and macrophages (MHC restriction). Class III loci are involved in complement fixation in the immune response (Klein 1979). Mapping of chromosome 17 has divided Class I genes into three regions (D,K, and L), Class II genes into two regions (A and E), and Class III genes into one region (S).

Because the MHC is highly polymorphic, it is difficult to characterize in wild populations. However, wild mice from at least 11 countries have been studied for MHC allele frequencies. From these studies, it has been estimated that the number of alleles at the Class I K and D regions is about 30, with mean allelic frequencies of 0.032 (H2-K) and 0.038 (H2-D). These estimates are expected to increase as sampling becomes more representative of global mouse populations (Klein 1986). Reagents are not yet available for identifying Class II molecules in these populations, but it is expected that the gene products of the A and E regions will prove to be as polymorphic as the Class I molecules.

The MHC in humans (HLA) is a highly polymorphic system, even in small, isolated populations (Black et al. 1981). The HLA was the third MHC system to be identified (after mouse and chicken). The human system is the model most often used in defining the functions of immunity and of the MHC. The human immune system is believed to be the most highly evolved of any species, and involves the

interactions of many different immunogobulins, enzymes, cells, and proteins. Originally, immunologists became interested in the MHC because of organ transplantation rejection. As research in transplantation has developed, so has the understanding of the MHC and its role in the immune system (Klein 1979).

One of the most important contributions resulting from HLA research is the association of MHC variability with disease susceptibility. HLA haplotypes are associated with Hodgkin's disease, Addison's disease, narcolepsy, multiple sclerosis, rheumatiod arthritis, and pernicious anemia (Cohen et al. 1984, Klein 1986). Continued research in human immunology should offer more information concerning the role of MHC in disease resistance and also may pose questions regarding its function in other species.

JUSTIFICATION AND OBJECTIVES

The MHC probably exists in all vertebrate species. On the basis of the foregoing discussion, it is clear that despite the central phylogenetic importance of fishes, their MHC has not been well investigated. Beyond the evolutionary interest, characterization of the MHC of fish has practical value as well. Mortality and morbidity due to disease account for major financial and economic losses in aquaculture. Estimates of the economic impact of diseases range from 10-15% to 30% of the cost of producing fish (Klontz 1985). Variable degrees of resistance and susceptibility to disease among fish stocks clearly exist, yet investigations of the genetic variation underlying disease resistance have not been undertaken (Chevassus and Dorson 1990).

Demonstration of genetic variability and linkage of marker alleles to differential disease resistance traits are necessary steps in determining effective programs of selection for improved disease resistance in aquaculture stocks (Hallerman and Beckman 1988). MHC gene products have been linked to immune responsiveness and disease resistance in a number of species (Briles 1960, Briles et al. 1977, Cohen et al. 1984, Chevassus et al. 1990). Knowledge of such linkages has been applied in selective breeding programs for traditional agriculture species (Briles 1960; Vaiman et al. 1986a, 1986b). The MHC has recently become an issue in the management of captive populations (Hughes 1991, Virjenhoek and Leberg 1991). However, the reagents and baseline data needed to distinguish MHC haplotypes, to link MHC markers and disease resistance traits, and to execute marker-assisted selection programs in aquaculture species are limited.

Immunogenetic variability in the chicken has been thoroughly investigated using serological techniques (Briles and Briles 1987). Standard antisera to distinguish allelic variants at the B-F and B-G loci of the chicken MHC have been developed. Agglutination of avian red blood cells by mammalian serum (Landsteiner et al. 1932) and of piscine cells by mouse antibodies (Shinohara et al. 1981) suggest that chicken anti-chicken antisera may provide a ready means of identifying piscine gene products specific to the MHC or other homologous antigen recognition systems.

Recent studies using allograft rejection and mixed leukocyte reactivity suggest that teleostean fish have a MHC (Chevassus and Dorson 1990, Stet et al. 1990, Stet and Egberts 1991). The goal of my research was to develop a more practical method for identifying MHC haplotypes in aquaculture species, using goldfish (*Carassius auratus*) as the model system. This species was chosen because prior studies demonstrated positive results in tissue graft rejection and immune recognition of lymphocyte surface immunoglobulins (Kallman 1964, 1970; Hildemann 1970) and because goldfish are easily maintained and manipulated in the laboratory.

By producing full-sib goldfish families, and utilizing their sera and erythrocytes for screening of MHC haplotypic variability, the following objectives of this study were:

1. To characterize MHC haplotypes using fish anti-fish antisera. By injecting goldfish with red blood cells (RBCs) from other goldfish, species-specific antibodies were produced for use in hemagglutination assays,

2. To characterize MHC haplotypes using chicken anti-chicken antisera to identify MHC glycoproteins on the surface of goldfish RBCs. Any action of the chicken antisera (B-F and B-G locus types) in hemagglutination assays would depend upon cross-reactivity between chicken and goldfish MHC glycoproteins,

3. To characterize MHC haplotypes using chicken anti-fish antisera. By injecting chickens with goldfish RBCs, antibodies specific to fish MHC haplotypes were produced for use in hemagglutination assays, and

4. To develop a fluorescent antibody assay for identifying MHC molecules on goldfish RBC surfaces using flow cytometry.

METHODS

Breeding and Culture of Goldfish

Comet variety goldfish broodstock were held at the aquaculture facility at Virginia Polytechnic Institute and State University (VPI & SU). Spontaneous ovulation of goldfish was carried out using methods of Razani et al. (1989a, 1989b), Stacy et al. (1979), and Michael Vaughan (Piedmont Fisheries,

Reidville, NC, pers. comm.) Progeny were reared in aquaria and fed a series of commercial feeds until reaching a size adequate for blood sampling (4-10 cm total length).

Blood Collection and Preservation

Blood samples were collected by heart, lateral line, or caudal vein puncture with heparinized 1 ml tuberculin syringes and 26 gauge needles. Blood was preserved in heparinized vacutainer tubes (Becton Dickenson). RBCs were separated from plasma through centrifugation (1000 rpm for 10 min) and washed 3 times in fish physiological saline (FPS) (Wolf 1963). Cells used in agglutination and flow cytometry assays were utilized within 7 days of collection. Plasma was stored at -20°C until needed.

An attempt was made at preserving RBCs in liquid nitrogen. Cells were immersed in 0.1 ml dimethyl sulfoxide (DMSO) and 0.9 ml FPS in cryovials and refrigerated 1 hour. Vials were then frozen at -70° C for 2-4 hours. After freezing, vials were placed in liquid nitrogen. Cells were then thawed rapidly (within 60 sec) in 37° C water bath and diluted with 10 ml of FPS.

Development and use of fish anti-fish antisera

Assessing immunogenetic variability of unknown extent in open breeding populations of goldfish can be simplified by determining the segregation of a limited number of haplotypic serotypes within the progeny of single pairings. Hence, full-sib families were produced through controlled matings, and serological reagents were prepared by injecting washed RBCs from each parent into the other.

Each parental goldfish was injected with 0.1 ml (approx 10^8) washed RBCs of the other parent. Injections were performed at weeks 0, 2, and 4. Ten days after the four week injection, antisera was drawn and frozen at -20°C. Parents were injected at three month intervals thereafter to boost antibody titers (S.A. Smith, VPI & SU, pers. comm.) Samples were collected and frozen over a 10 month period. Pooled samples of these antisera were used for aggutination and for flow cytometry assays.

In order to eliminate interference from non-specific antibodies, pooled antisera from each parent was absorbed with pooled RBCs of goldfish from other families. Absorptions were performed by using equal volumes of antisera to diluted RBCs (dilutions no higher than 1:4 total volume). Each antisera was absorbed six times by setting up a series of six tubes of RBCs and incubating the antisera for 30 min (unless hemolysis occurred) in each tube. At the end of each 30 min interval, the tubes were centrifuged at 1000 rpm for 5 min to separate the cells from the antisera (W.E. Briles, Northern Illinois University (NIU), pers. comm.). After absorption, the antisera was stored at -20° C.

Fish anti-fish antisera was used to test progeny RBCs by agglutination. Agglutination tests were performed by mixing 10 ul of progeny cells (washed three times) with 50 ul of each parental antisera in glass tubes. Tubes were examined at approximately 90 min for agglutination. Final results were confirmed by overnight incubation at 4°C. Weak or negative results were confirmed by observation with a compound microscope.

Use of chicken anti-chicken antisera

Six system-specific antisera for variants at the chicken B-F and B-G loci (Briles et al. 1948) were provided by Dr. E. W. Briles of NIU. These antisera (diluted 1:4) were used to screen full sib families of goldfish in agglutination assays similar to those described for fish anti-fish antisera. The chicken anti-chicken antisera used represented the following haplotypes at the chicken B locus: #597 = B^{19}

B^{19}, #361 = B^{24} B^{24}, #147 = B^{19} B^{19}, #146 = B^{23} B^{24}, #538 = B^{23} B^{24}, #585 which contained both B^{23} B^{24} and B^{24} B^{24}.

Development and use of chicken anti-fish antisera

RBCs collected from goldfish parents were shipped to the laboratory of Dr. Briles. Goldfish blood (approx. 1 ml) was mixed with FPS (2 ml) and injected into chickens (a different chicken for each goldfish sample) once a month for three months. After three months, the chickens were bled and the antisera were sent to VPI & SU for absorption and testing. Absorptions were performed as described above to remove nonspecific antibodies. RBCs of progeny in each goldish family were tested by agglutination (described above) using the chicken anti-fish antisera made from cells of parental cells for that family.

Development of fluorescent antibody assay

Because it proved difficult to obtain adequately large blood samples from small goldfish for a battery of conventional agglutination tests, an attempt was made to develop an alternative method using smaller samples to determine MHC phenotypes using flow cytometry. Currently there are no commercially available fluorescent antibodies that are specific to goldfish IgM. Therefore, in order to test for agglutination using the flow cytometer, a second antibody approach was used to bind fluorescent antibodies to fish antisera. First, goldfish Ig was isolated for production of rabbit anti-goldfish IgM. The process involved: (1) the injection of TNP-keyhole hemocyanin (TNP-KLH) antigen into goldfish to evoke expression of high levels of anti-TNP antibodies, (2) haptenation of

Sephrose 4B with trinitrobenzyl sulfonic acid in order to determine if anti-TNP antibodies were present in the goldfish antisera to TNP-KLH, (3) and affinity purification of the goldfish Ig. After the Ig was isolated, two male rabbits were injected with the goldfish IgM to produce rabbit anti-goldfish IgM. Then rabbit anti-goldfish antiserum was used to bind the fluorescent goat anti-rabbit IgG used in the flow cytometry procedure.

Isolation of Goldfish IgM

Four large goldfish (400-550g) were immunized interperitoneally (IP) with 50 ul trinitrophenylated keyhole limpet hemocyanin (TNP-KLH) antigen (donated by Dr. Norman Miller, University of Mississippi Medical Center) emulsified (1:1) in Freund's complete adjuvant (Sigma) (Miller et al. 1984). After 28 days, the immune response of the fish was boosted with TNP-KLH emulsified in Freund's incomplete adjuvant (Sigma). Two weeks later, the fish were bled to collect serum, and the anti-hapten titer was determined by hemagglutination assay using TNP-sheep red blood cells (red blood cells donated by the Virginia-Maryland Regional College of Veterinary Medicine at VPI & SU).

Haptenation of SRBC with Trinitrobenzyl Sulfonic Acid (TNBS)

Sheep red blood (SRBCs) cells were washed three times in cold PBS, and were pelleted by centrifugation at 2100 rpm between each wash. A 50 ml beaker was wrapped in foil to protect reagents from light, and 60mg of trinitrobenzyl sulfonic acid (TNBS) was placed in the beaker. Twenty-one ml of cacodylate buffer (9.55g cacodylic acid / 250 ml PBS) was added to the TNBS (Miller et al. 1984). Three ml of SRBCs was added to the solution and placed on a shaker for 15 min. PBS was then added to bring total volume to 45 ml and mixed gently. The solution was centrifuged at 2100 rpm for five min, and the supernatant was removed by aspiration. Thirty-five ml of cold PBS (containing 22 mg of glycl-glycine) was added to the SRBCs. The solution was centrifuged as above, and the supernatant was removed by aspiration. The cell dilution was then adjusted to 1:100 using

PBS. This solution was then used to determine the antibody titer using a microtiter plate. Twenty ul of TNP-KLH serum, 20 ul of PBS, and 20 ul of haptenated SRBCs were used in the procedure. Dilutions were made up to 1:4168. The last positive dilution was 1:2084. Since titers of 1:2000 or greater are considered very significant for this procedure, the fish were bled at 2 week intervals (1 ml each bleed) for six weeks. The fish were given one additional boost of TNP-KLH and Freund's incomplete adjuvant to assure that titers remained at at least 1:2000.

IgM Affinity Purification

Purification of the IgM antibody was necessary before injection of the antibody into rabbits for production of rabbit anti-goldfish IgM. A Sephrose 4B column (Sigma 4B-200) was filled with 10 ml of beads conjugated with dinitrophenol lysine. The beads were washed five times with PBS (a beaker was placed under the column for collecting samples), and then the serum (3-5 ml pooled from the four goldfish) was added and allowed to pass through the column by gravity filtration. After the serum passed through the column, a PBS wash was added and allowed to mix with the serum. The beaker containing eluate was removed and a clean beaker was placed under the column. Nine ml (1.5 column volumes) of hapten (DNP-OH) was passed through the column to elute the antibody, followed by one wash of PBS. This was the sample containing the IgM-like molecules for injection into rabbits.

The hapten eluted sample was then placed in dialysis tubing and the tubing was immersed in PBS to remove the DNP hapten. The sample was incubated overnight, and the PBS was changed two times the following day. The antibody sample was then concentrated by centrifugation at 5000 rpm, at 4°C, for 30 min. Identification and specificity of the IgM antibody was then tested by use of polyacrylamide gel electrophoresis and Ouchterlony plates (Fisher).

Ouchterlony plates were used to assay the presence of anti-goldfish IgM from two rabbits immunized with goldfish antibody. Prior to the injections with goldfish antibody, each rabbit was bled for a preimmune sample. The samples that were added to the wells of the plate included: (1) serum

sample from a rabbit #818 after injection with TNP-KLH, (2) serum from rabbit #818 before injections with TNP-KLH, (3) serum sample from rainbow trout (*Onchornychus mykiss*), (4) serum sample from hybrid tilapia (*Sarotherodon niloticus x S. aureus*), (5) serum sample from hybrid striped bass (*Morone chrysops x M. saxatilis*), (6) a sample of PBS as a negative control, (7) and a serum sample of a randomly selected goldfish not previously injected with TNP-KLH.

Injection of Rabbits with Goldfish Ig

Two male rabbits were injected with a mixture of 0.5 ml goldfish Ig and 0.5 ml Freund's complete adjuvant (0.5 ml subcutaneously near the shoulder blades, 0.5 ml IM in leg). After 14 days, each was again injected with 0.5 ml goldfish Ig and 0.5 ml Freund's incomplete adjuvant. The injection was repeated at day 21, and then a small test bleed (5 ml) was taken for quantification of protein titers. On day 26, each rabbit was bled (30 ml). The serum was separated from the cells, incubated at 56°C, and then absorbed with goldfish RBCs as described in the development of fish anti-fish antisera. Serum was then frozen until needed.

Testing of Goldfish RBCs Using Flow Cytometry

Individual tubes were labeled for each family member, with a separate tube for each type of antisera. The panel of antisera used included fish anti-fish, chicken anti-chicken, and chicken anti-fish reagents for each family. RBCs (10 ul) of individual family members were incubated 30-45 min in 50 ul of each antisera. After the incubation with the antisera panel, cells were washed three times in PBS and then incubated in 1 ml rabbit anti-goldfish IgM antisera (1:500 dilution) for 30-45 min. Cells were again washed three times and then incubated 30-35 min in 1 ml of fluorescein (DTAF)-conjugated affinipure rabbit anti-chicken IgG (Jackson ImmunoResearch Laboratories, Inc.). Cells were washed three times and then were suspended in 1 ml FPS for sampling in the flow cytometer (Coulter Epics

V 752). The wavelength of the laser was set at 488 nm. Fluoresence detected by the flow cytometer was analyzed by computer software provided by the manufacturer of the machine (Coulter MDADS).

RESULTS

RBC preservation

The RBCs preserved in liquid nitrogen did not remain intact. It appeared that hemolysis occured during the the rapid thawing stage of the procedure. The integrity of the RBCs was compromised to the extent that it was not possible to use them for hemagglutination assays.

Agglutination assays

Antisera panel

Washed RBCs collected from seven goldfish families were screened for agglutinations with fish anti-fish, chicken anti-chicken, and chicken anti-fish antisera. RBCs for broodstock (identified by letters and numbers) and progeny (identified by numbers only) were tested individually with the following panel of antisera, which will be referred to by numbers in the text and tables to follow:

1. Father fish-anti fish
2. Mother fish anti-fish
3. Chicken anti-chicken #597
4. Chicken anti-chicken #361
5. Chicken anti-chicken #147
6. Chicken anti-chicken #146

7. Chicken anti-chicken #538

8. Chicken anti-chicken #585

9. Father chicken anti-fish

10. Mother chicken anti-fish

Antisera 1, 2, 9, and 10 were specific to each family tested. RBCs from each parent were analyzed with plasma from its mate before injections of RBCs began. All results were negative prior to these injections. The following sections present results of the agglutination assays for RBCs from members of goldfish families tested with fish anti-fish, chicken anti-chicken, and chicken anti-fish antisera.

D102 x M103 full-sib family #1

Antisera #1 and #2 for this family were used as controls for D102 (father)and M103 (mother), respectively. Antisera contained plasma collected before the initiation of the injection series used to produce the fish-anti fish antisera. Of the RBCs from 20 F_1s tested with the fish anti-fish antisera produced by the father (against the antigens of the mother), 11 assays were positive (Table 1). There were 20 positive results of RBCs of progeny with the mother fish anti-fish antiserum produced against the father antigens. Assays involving RBCs from seven of these F_1s were positive to both parental fish antifish anti-sera, and six were negative to both parental antisera. These data indicate that the cross-injections between broodstock were successful in producing antibodies that could be detected by surface antigens on the RBCs of the F_1s. If the parents had shared the same RBC surface antigens, one would have expected positive reactions of 100%.

With the chicken anti-chicken antisera, RBCs from 15 family members exhibited agglutination to #597, 13 were positive to #361, nine were positive to #147, 19 were positive to #146, 22 were positive to #538, and 10 were positive to #585.

RBCs for different family members differed in terms of the patterns of presence or absence of agglutinations against the panel of antisera. Looking across the rows of Table 1, it is clear that certain patterns of reactivity or non-reactivity were shown among family members. Distinctive patterns of reactivity were designated as agglutination phenotypes. These phenotypes were taken as indicative of underlying genotypic variability at the genetic loci encoding receptor molecules on the surface of RBCs. Thus, with the panel of chicken anti-chicken antisera, 12 different phenotypes were observed. Phenotype #5 for this family was observed more often than any other, exhibited by four individuals.

Use of the chicken anti-fish antisera led to positive reactions with RBCs from all 22 fish tested with the father-derived chicken anti-fish antisera. There were 21 positive reactions with the mother-derived chicken anti-fish antisera.

J221 x C222 full-sib family #2

Antisera #1 and #2 for this family were used as controls for J221 and C222. (broodstock). Of the RBCs from the seven F_1s tested with father fish anti-fish antisera, six were positive (Table 2). RBCs from the same six fish showed positive results with the mother fish anti-fish antisera. Assays of RBCs from only one F_1 was negative to both parental antisera.

With the chicken anti-chicken antisera, RBCs from three family members exhibited positive agglutination results to #597, four were positive to #361, three were positive to #147, eight were positive to #146, eight were positive to #538, and two were positive to #585. From this family of nine individuals, six phenotypes were identified with the chicken anti-chicken antisera. Phenotype #2 for this family was observed more often than any other, exhibited by three individuals.

Agglutinations of the chicken anti-fish antisera were positive with RBCs of all but one F_1, which was negative to both the father chicken anti-fish and the mother chicken anti-fish antisera.

Table 1. Agglutination assays of family #1 (D102 & M103). The letter F identifies RBCs tested in each family. The antisera panel consisted of fish anti-fish (1 & 2), chicken anti-chicken (3-8), and chicken anti-fish antisera (9 & 10). Column 11 presents the phenotypic designation. The symbol + indicates positive agglutination results with RBCs and antisera. The symbol - indicates negative agglutination results with RBCs and antisera.

F	1	2	3	4	5	6	7	8	9	10	11
D102	-	+	+	-	+	-	+	-	+	+	1
M103	+	-	-	-	-	+	+	+	+	+	2
109	+	-	-	-	-	+	+	-	+	+	3
110	+	-	+	-	+	+	+	+	+	+	8
111	-	+	+	+	-	+	+	-	+	+	4
112	-	-	-	-	-	+	+	-	+	+	3
113	+	+	+	+	-	+	+	+	+	+	5
114	+	-	+	-	-	-	+	+	+	+	6
115	-	+	+	+	+	+	+	-	+	+	12
116	-	-	+	+	+	+	+	-	+	+	7
117	-	+	+	+	+	+	+	+	+	+	9
118	+	+	+	-	+	+	+	-	+	+	10
119	+	+	+	+	+	+	+	+	+	+	9
120	+	-	-	+	-	-	+	-	+	+	11
121	-	-	-	-	-	+	+	-	+	+	3
122	+	+	+	+	-	+	+	-	+	+	4
123	-	-	+	+	-	+	+	-	+	+	4
124	+	+	+	+	-	+	+	+	+	+	5
125	+	+	+	-	+	+	+	+	+	+	8
126	+	+	-	+	+	+	+	-	+	+	7
127	-	-	-	+	-	+	+	+	+	+	5
128	-	-	+	+	-	+	+	+	+	+	5

Table 2. Agglutination assays of family #2 (J221 & C222). The letter F identifies RBCs tested in each family. The antisera panel consisted of fish anti-fish (1 & 2), chicken anti-chicken (3-8), and chicken anti-fish (9 & 10) antisera. Column 11 presents the pheontypic designation. The symbol + indicates positive agglutination results with RBCs and antisera. The symbol - indicates negative agglutination results with RBCs and antisera.

F	1	2	3	4	5	6	7	8	9	10	11
J221	-	+	+	+	+	-	+	-	+	+	1
C222	+	-	+	-	+	-	+	+	+	+	2
201	+	+	-	-	-	+	+	-	+	+	3
202	-	-	-	+	+	+	-	-	-	-	4
203	+	+	-	-	-	+	+	-	+	+	3
204	+	+	-	+	-	+	+	-	+	+	5
205	+	+	-	-	-	+	+	-	+	+	3
206	+	+	-	+	-	+	+	-	+	+	5
207	+	+	+	-	-	+	+	-	+	+	6

B321 x B322 full-sib family #3

Antisera #1 and #2 for this family were used as controls for B321 and B322 (broodstock). Of the RBCs from the 20 F_1s tested with father fish-anti fish antisera, 19 were positive (Table 3). Assays of RBCs from twenty of the F_1s tested with the mother fish anti-fish antisera were positive.

Of RBCS from the 22 fish tested with the chicken anti-chicken antisera, 13 family members exhibited positive agglutination to #597, five were positive to #361, eight were positive to #147, 19 were positive to #146, 18 were positive to #538, and eight were positive to #585. In this family, 13 phenotypes were identified with chicken anti-chicken antisera. Phenotype #5 for this family was observed more often than any other, exhibited by five individuals.

The chicken anti-chicken antisera showed positive reactions with RBCs from all 22 family members.

F421 x W422 full-sib family #4

Antisera #1 and #2 for this family were used as controls for F421 and W422 (broodstock). All of the RBCs from the 20 F_1s tested were positive to both the father fish anti-fish and mother fish anti-fish antisera (Table 4).

Of the RBCs from the 22 fish analyzed from this family with chicken anti-chicken antisera, six were positive to #597, nine were positive to #361, six were positive to #147, 14 were positive to #146, seven were positive to #538, and five were positive to #585. Fifteen different phenotypes were identified. Phenotype #5 for this family was observed more often than any other, exhibited by three individuals.

Due to the late spawn, no chicken anti-fish antisera were prepared for this family.

Table 3. Agglutination assays of family #3 (B321 & B322). The letter F identifies RBCs tested in each family. The antisera panel consisted of fish anti-fish (1 & 2), chicken anti-chicken (3-8), and chicken anti-fish (9 & 10) antisera. Column 11 presents the phenotypic designation. The symbol + indicates negative agglutination results with RBCs and antisera. The symbol - indicates negative agglutination results with RBCs and antisera.

F	1	2	3	4	5	6	7	8	9	10	11
B321	-	+	+	+	+	+	-	+	+	+	1
B322	+	-	+	+	-	+	+	+	+	+	2
301	+	+	-	-	+	+	+	-	+	+	3
302	+	+	-	-	+	+	+	-	+	+	3
303	+	+	+	+	+	+	-	+	+	+	1
304	+	-	-	-	-	+	+	-	+	+	4
305	+	-	-	-	-	+	+	-	+	+	4
306	+	-	-	-	-	+	+	-	+	+	4
307	+	+	+	-	-	+	+	-	+	+	5
308	+	-	+	-	-	+	-	-	+	+	6
309	+	+	-	-	-	+	+	-	+	+	4
310	+	-	-	-	+	-	+	-	+	+	7
311	+	+	+	-	-	+	+	-	+	+	5
312	+	+	+	-	-	+	+	-	+	+	5
313	+	+	+	+	-	+	+	+	+	+	8
314	+	+	+	-	-	+	+	-	+	+	5
315	-	+	+	+	+	+	+	+	+	+	9
316	+	+	-	-	-	-	+	-	+	+	10
317	+	+	-	-	+	+	+	+	+	+	11
318	+	+	+	-	+	+	+	+	+	+	12
319	+	+	+	-	-	+	+	-	+	+	5
320	+	+	+	-	-	+	-	+	+	+	13

Table 4. Agglutination assays of family #4 (F421 & W422). The letter F identifies RBCs tested in each family. The antisera panel consisted of fish anti-fish (1 & 2), and chicken anti-chicken antisera (3-8). Column 9 presents the phenotypic designation. The symbol + indicates positive agglutination results with RBCs and antisera. The symbol - indicates negative agglutination results with RBCs and antisera.

F	1	2	3	4	5	6	7	8	9
F421	-	+	+	+	+	+	+	+	1
W422	+	-	+	-	+	+	+	+	2
401	+	+	-	-	+	+	-	-	3
402	+	+	-	+	-	-	-	-	4
403	+	+	-	-	-	+	-	-	5
404	+	+	+	+	+	+	+	+	1
405	+	+	+	+	-	-	-	-	6
406	+	+	-	+	-	+	-	-	7
407	+	+	+	-	-	-	+	-	8
408	+	+	-	-	+	+	-	-	3
409	+	+	-	+	-	+	+	-	9
410	+	+	+	-	-	-	-	-	10
411	+	+	-	-	-	+	+	-	11
412	+	+	-	+	-	+	+	-	9
413	+	+	-	+	-	+	-	-	7
414	+	+	-	-	-	-	-	-	12
415	+	+	-	+	-	+	-	+	13
416	+	+	-	-	-	+	-	-	5
417	+	+	-	-	-	+	-	-	5
418	+	+	-	-	-	-	-	-	12
419	+	+	-	-	-	-	-	+	14
420	+	+	-	-	+	-	-	-	15

N521 x J522 full-sib family #5

Antisera #1 and #2 for this family were used as controls for N521 and J522 (broodstock). Of the RBCs from the 20 F_1s analyzed in this family, 11 were positive to the father fish-anti fish antisera and 20 were positive to the mother fish anti-fish antisera (Table 5).

With the chicken anti-chicken antisera, RBCs from eight family members were positive to #597, 13 were positive to #361, 11 were positive to #147, 13 were positive to #146, 14 were positive to #538, and 10 were positive to # 585. Seventeen phenotypes were identified with the chicken anti-chicken antisera. (There are no results recorded for J522 for chicken anti-chicken antisera because of mortality of the mother of the family).

All assays involving RBCs tested with the father chicken anti-fish and mother chicken anti-fish antisera were positive.

P621 x D622 full-sib family #6

Antisera #1 and #2 for this family for P621 were used as controls. There were no negative controls run for D622 because of mortality of the fish before antiserum was prepared. Of the RBCs from the 20 fish tested with mother fish anti-fish antisera, all 20 gave positive results (Table 6).

With the chicken anti-chicken antisera, RBCs from nine family members exhibited agglutination positive to #597, five were positive to #361, 10 were positive to #147, nine were positive to #146, 10 were positive to #538, and nine were positive to #585. Nineteen phenotypes were identified with the chicken anti-chicken antisera.

There were no chicken anti-fish antisera made for this family due to mortalities of P621 & D622.

Table 5. Agglutinations of family #5 (N521 & J522). The letter F identifies RBCs tested in each family. The antisera panel consisted of fish anti-fish (1 & 2), chicken anti-chicken (3-8), and chicken anti-fish (9 & 10) antisera. Column 11 presents the phenotypic designation. The symbol + indicates positive agglutination results with RBCs and antisera. The symbol - indicates negative agglutination results with RBCs and antisera.

F	1	2	3	4	5	6	7	8
501	33.1	49.9	19.2	50.2	31.5	64.3	29.2	29.3
502	13.4	5.7	35.3	23.3	28.7	36.7	43.5	15.9
503	4.1	34.7	18.9	34.7	53.9	47.7	26.4	20.9
504	3.9	41.1	20.2	18.4	28.2	41.5	12.7	20.6
505	8.7	10.1	19.6	24.9	34.3	47.8	13.0	18.8
506	5.6	13.9	7.0	10.4	7.5	10.0	6.6	15.5
507	9.6	39.2	3.3	39.4	6.9	12.2	2.2	4.0
508	6.8	23.4	8.0	23.8	38.9	11.3	5.3	7.5
509	17.6	40.3	3.7	27.6	28.0	4.5	1.8	6.7
510	4.5	5.0	22.4	12.4	61.8	21.1	25.1	19.9
511	1.8	7.8	5.8	7.6	9.5	24.1	18.2	16.3
512	2.1	10.4	6.2	4.7	5.6	6.8	4.5	7.7
513	2.6	3.1	9.8	4.2	18.0	18.6	13.3	28.2
514	13.3	13.1	10.8	5.9	13.6	18.7	11.2	20.3
515	20.3	8.4	16.4	5.4	7.5	25.7	13.3	43.2
516	4.0	15.7	5.0	7.0	38.8	36.7	22.9	37.3
517	15.6	26.4	13.7	17.4	41.3	41.3	14.2	30.9
518	3.4	18.7	25.4	24.3	42.4	42.3	11.9	41.8
519	5.5	19.7	32.1	14.8	31.6	7.5	34.9	47.8
520	4.1	3.9	14.7	10.6	30.5	38.3	6.9	27.2

Table 6. Agglutination assays of family #6 (P621 & D622). The letter F identifies RBCs tested in each family. The antisera panel consisted of fish anti-fish (1 & 2), and chicken anti-chicken antisera (3-8). Column 9 presents the phenotypic designation. The symbol + indicates positive agglutination results with RBCs and antisera. The symbol - indicates negative agglutination results with RBCs and antisera.

F	1	2	3	4	5	6	7	8	9
P621		+	+	-	-	+	-	-	1
D622									
601		+	+	-	-	+	-	+	2
602		+	+	-	-	-	-	+	3
603		+	+	+	+	+	+	+	4
604		+	+	-	+	+	+	-	5
605		+	+	-	+	-	-	-	6
606		+	-	-	-	-	+	+	7
607		+	-	-	+	-	+	+	8
608		+	-	+	-	+	-	+	9
609		+	+	-	-	-	+	-	10
610		+	-	-	-	+	+	-	11
611		+	-	+	-	+	+	+	12
612		+	+	-	-	-	+	-	10
613		+	-	+	+	-	+	+	13
614		+	-	-	-	-	-	-	14
615		+	-	-	+	+	-	-	15
616		+	-	-	+	-	-	-	16
617		+	-	-	-	-	+	+	17
618		+	-	-	+	-	+	-	18
619		+	+	+	+	+	-	-	19
620		+	-	-	+	-	-	-	6

J721 x Y722 full-sib family #7

Antisera #1 and #2 for this family were used as controls for J721 and Y722. Of the RBCs from the 20 F_1s tested, 12 were positive to the father fish anti-fish antisera, and 11 were positive to the mother fish anti-fish antisera (Table 7). Five fish were positive to both the father and mother fish anti-fish antisera, and two were negative to both.

With the chicken anti-chicken antisera, RBCs from four family members were positive to #597, 14 were positive to #361, five were positive to #147, 10 were positive to #146, none of the fish were positive to #538, and eight were positive to #585. Twelve phenotypes were identified. Phenotype #9 for this family was observed more often than any other, exhibited by three individuals.

There were no chicken anti-fish antisera made for this family due to a late spawn.

Table 7. Agglutination assays of family #7 (J721 & Y722). The letter F identifies RBCs tested in each family. The antisera panel consisted of fish anti-fish (1 & 2), chicken anti-chicken antisera (3-8). Column 9 presents the phenotypic designation. The symbol + indicates positive agglutination results with RBCs and antisera. The symbol - indicates negative agglutination results with RBCs and antisera.

F	1	2	3	4	5	6	7	8	9
J721	-	+	+	+	+	+	-	+	1
Y722	+	-	+	+	-	-	-	-	2
701	-	-	-	-	-	-	-	-	3
702	+	-	-	+	+	+	-	-	4
703	+	+	+	+	+	+	-	+	1
704	+	+	-	+	-	-	-	+	5
705	+	-	-	-	+	-	-	-	6
706	+	+	-	+	+	+	-	-	4
707	+	-	-	-	-	-	-	-	3
708	-	+	-	+	-	+	-	-	7
709	-	+	-	-	-	-	-	+	8
710	+	-	-	+	-	-	-	-	9
711	+	-	-	-	-	+	-	-	10
712	-	+	-	-	-	-	-	+	8
713	-	-	-	+	-	-	-	-	9
714	-	+	-	-	-	-	-	-	3
715	-	+	-	+	-	+	-	+	11
716	-	+	-	-	-	+	-	-	10
717	+	-	-	+	-	+	-	+	11
718	+	-	-	+	-	+	-	-	7
719	+	+	+	+	-	-	-	+	12
720	+	+	-	+	-	-	-	-	9

Agglutination phenotypes considered within and between families

In this study, RBCs from 139 fish from seven full-sib families were tested against the antisera panel for agglutination. Comparisions of patterns of reactivity revealed a number of specific phenotypes segregating both within and between families.

For diploid organisms, one would expect each parent to transmit two MHC haplotypes to the progeny, thereby giving rise to as many as four MHC haplotypes among the progeny of a single pair mating. Assuming a one-to-one correspondence of MHC haplotypes and agglutination phenotypes, a correspondingly limited number of agglutination phenotypes was expected within full-sib families. However, more agglutination phenotypes were observed among the data for given families than was expected under the model. Two explanations for these results seem likely. It is possible that not all non-specific antibodies were absorbed out in production of the respective antisera, giving rise to more positive reactions than might have occured following more absorptions. Another explanation is that because goldfish are tetraploid organisms, activities of duplicated loci allowed more combinations of haplotypic inheritance to be expressed. Data collected in this study did not allow testing of these two hypotheses.

With the fish anti-fish antisera, both positive and negative patterns of reactivity were observed in each family. This reactivity implies that the cross-injections between broodstock was successful in stimulating an antibody response to surface antigens of the parent inherited by the progeny. In most of the families, positive reactions to both parental antisera were observed in most cases (Tables 1-8). The positive reactions could have been a result of the large number of phenotypic combinations possible in this species, as explained above.

Results of agglutination with the chicken anti-chicken antisera showed many possible phenotypes within and between families. A total number of 48 phenotypes was observed. The

phenotype observed most often, -, -, -, +, +, and -, against the panel of chicken anti-chicken antisera, was observed in a total of 17 individuals from five of the families. Observation of particular agglutination phenotypes among families was taken as indication of segregation of common MHC haplotypes among the respective families. The number of phenotypes observed overall suggested a rather high level of genetic diversity at loci affecting expression of relevant surface antigens in the collection of goldfish utilized. Antiserum #146 reacted with more individuals than any other, with 92 positive reactions (66%) out of 139. This reagent was designated as B^{24} B^{24} in the chicken system.

Use of the chicken anti-fish antisera yielded positive results for all progeny tested in every family, with the exception of one individual (Table 2). The high frequency of positive results suggests that either many more absorptions were needed to produce antisera useful in distinguishing genetic variability, or that so many antigens and receptor systems were active that production of such antisera was unlikely.

Development of fluorescent antibody assays

A fluorescent antibody assay was developed for testing goldfish RBCs for the presence or absence of agglutination against members of a panel of antisera. In order to bind a fluorescence-marked rabbit antibody to a given serum, rabbit anti-goldfish Ig was needed, giving rise to the need for isolation of goldfish IgM.

Isolation and characterization of goldfish IgM.

Goldfish antibody was isolated by affinity purification utilizing the haptenation procedure previously described. Observation of reactivity on Ouchterlony plates (Figure 1) indicated that goldfish Ig had indeed been isolated. Molecular weight of the presumptive IgM molecule was determined by polyacrylamide gel electorphoresis (Figure 2). Migration distances observed showed that the molecular weight of the light chain was in the range of 20.5 -24.0 kD, and of the heavy chain in the range of 72.0-81.0 kD. These molecular weights agreed with those observed by Marchalonis (1971).

Use of flow cytometry to test reactivity against the antisera panel

Flow cytometry was used as a means of testing the binding of RBCs tagged with a fluorescent rabbit anti-goldfish IgM against members of the panel of antisera. Data was recorded in percentages of total fluorescence emitted by the RBC population that did bind antibody for each individual tested (Figure 3). The results of those tests for RBCs from members of goldfish families 2, 3, 4, and 5 are presented in Tables 8, 9, 10, and 11, respectively.

Figure 1. Identification of presumptive goldfish IgM. Ouchterlony plates were used to assay the presence of goldfish IgM that was injected into two rabbits to produce rabbit anti-goldfish IgM. Reactivities of seven serum samples against the Ig sample were tested. The sample in test well #1 was serum from rabbit #818 after injection of TNP-KLH; #2, serum from rabbit #818 before injections of TNP-KLH; #3, serum from rainbow trout; #4, serum from tilapia; #5, serum from hybrid striped bass;, #6, PBS control; and #7, serum from a goldfish selected at random. The positive reaction between the post Igm serum (#1) and the goldfish serum in #7 indicated the presence of the goldfish Ig.

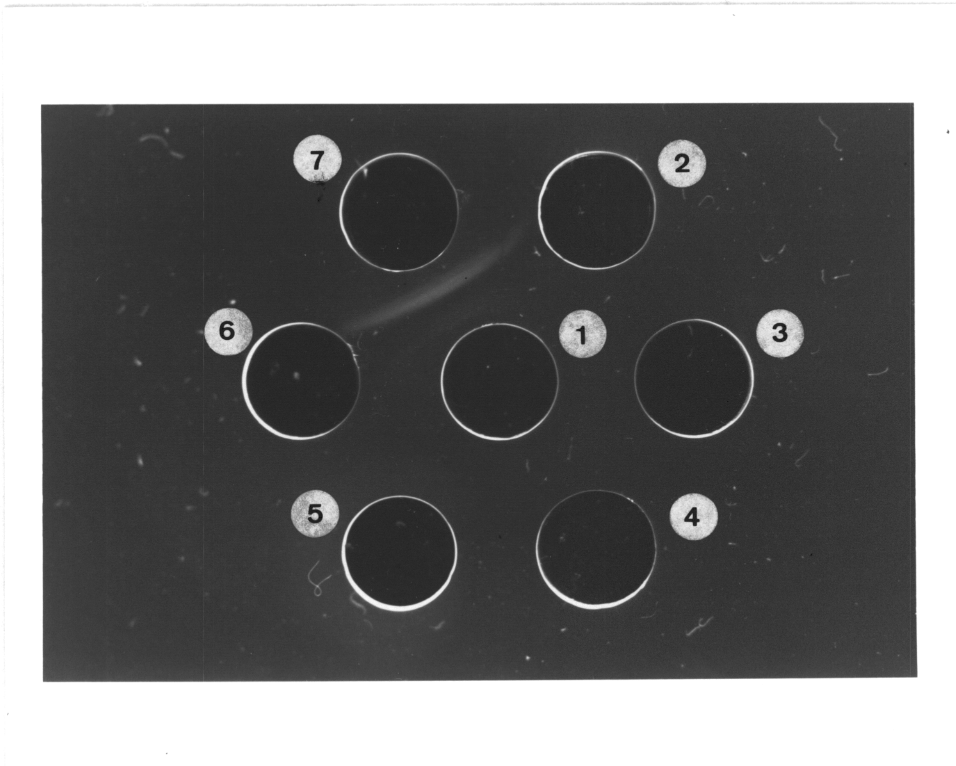

Figure 2. Molecular weight determination for presumptive goldfish IgM. The first lane in this polyacrylamide gel contains the sample reduced with 2 beta-mercaptoethanol, lane two contained the non-reduced sample, and lane three contains the six size standards, specified in kD.

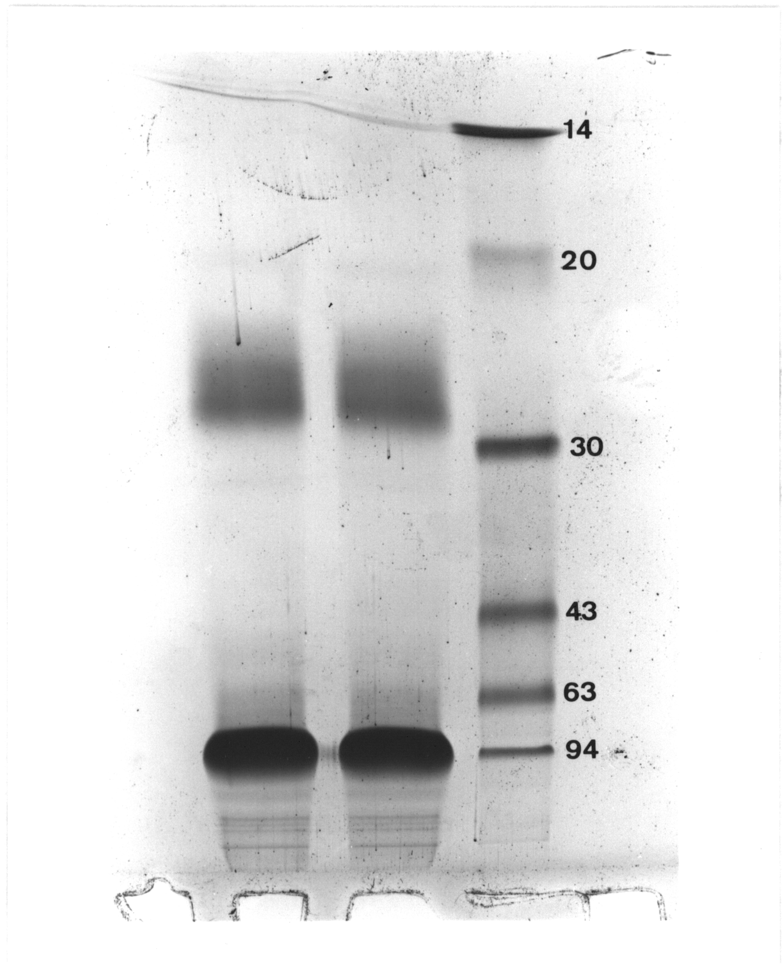

Figure 3. Positive binding of fluorescently tagged antibodies by goldfish RBCs. The two peaks represent populations of cells not binding (left) and binding (right) to the fluorescence tagged antibody. The histogram on the left illustrates negative reactivity with antiserum #8 and RBCs from fish 205. The histogram on the right illustrates positive results with antisera #10 and RBCs from fish 205.

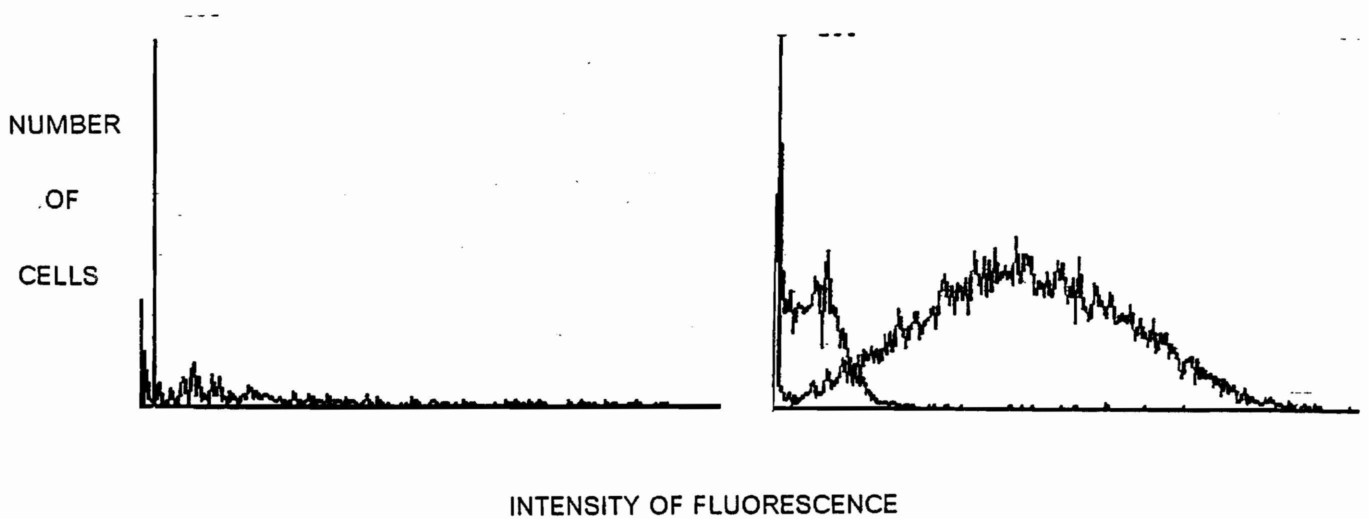

Table 8. Flow cytometry results of family #2 (J221 & C322). Reactivities of goldfish RBCs with fluorescence-tagged antibodies are given. The letter F identifies individuals in each family where RBCs were used in reactivity assays. The antisera panel consisted of family-specific fish anti-fish (columns 1 & 2), chicken anti-chicken (columns 3-8), and chicken anti-fish (columns 9 & 10) antisera. The results recorded in each column are percentages of fluorescence tagged antibodies bound by the goldfish RBCs as measured by the flow cytometer.

F	1	2	3	4	5	6	7	8	9	10
201	55.6	88.5	24.5	75.7	59.8	65.3	10.8	9.5	41.8	83.3
202	0.0	94.8	33.5	80.0	71.4	75.3	24.1	24.7	70.3	74.8
203	79.3	82.7	19.9	22.3	33.8	14.5	5.9	12.9	54.2	66.1
204	89.6	91.0	31.7	53.0	59.9	60.2	27.1	9.3	49.7	80.0
205	4.7	78.1	18.3	51.9	56.6	60.0	5.4	8.1	43.4	87.0
206	80.0	70.8	38.9	66.5	63.8	62.4	10.6	10.4	34.6	90.3
207	68.6	61.5	37.1	66.0	61.1	74.7	14.9	15.3	58.8	47.2

Table 9. Flow cytometry results of family #3 (B321 & B322). The letter reactivities of goldfish RBCs with fluorescence-tagged antibodies are given. The letter F identifies individuals in each family where RBCs were used in reactivity assays. The antisera panel consisted of family-specific fish anti-fish (columns1 & 2), and chicken anti-chicken (columns 3-8) antisera. The results recorded in each column are percentages of fluorescence-tagged antibodies bound by the goldfish RBCs as measured by the flow cytometer.

F	1	2	3	4	5	6	7	8
301	76.4	0.0	30.4	0.0	11.5	44.3	0.0	12.7
302	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
303	5.5	4.9	0.0	20.8	34.5	4.7	27.1	16.8
304	20.7	2.4	29.4	7.3	36.9	64.3	9.6	50.5
305	0.0	5.2	19.0	7.5	21.0	17.5	16.5	9.0
306	2.7	3.6	8.2	12.9	18.9	8.0	3.3	5.7
307	0.0	6.2	16.1	11.1	6.1	41.3	4.2	3.7
308	16.9	0.0	0.0	15.2	22.9	31.5	0.0	16.3
309	0.0	0.0	0.0	7.5	0.0	0.0	0.0	0.0
310	12.4	33.9	14.4	12.2	12.0	0.0	0.0	0.0

Table 10. Flow cytometry results of family #4 (F421 & W422). Reactivities of goldfish RBCs with fluorescence-tagged antibodies are given. F identifies individuals in each family where RBCs were used in reactivity assays. The antisera panel consisted of fish anti-fish (1 & 2), and chicken anti-chicken (3-8) antisera. The results recorded in each column are percentages of fluorescence tagged antibodies bound by the goldfish RBCs as measured by the flow cytometer.

F	1	2	3	4	5	6	7	8
401	4.8	3.6	26.3	5.2	22.0	61.6	27.2	25.2
402	8.2	10.3	23.1	11.7	31.8	40.7	27.7	11.2
403	15.3	10.8	11.8	9.6	25.5	30.6	12.1	7.5
404	7.8	12.1	30.8	13.9	38.3	66.4	36.5	52.4
405	10.7	11.6	22.8	13.9	39.8	65.8	55.4	45.6
406	9.5	7.9	17.3	8.7	19.5	42.9	26.5	4.3
407	3.4	7.4	38.2	21.5	53.1	63.1	59.2	65.8
408	1.4	4.6	25.6	10.0	29.1	57.4	43.4	48.7
409	14.7	14.2	24.9	20.1	34.2	66.1	70.3	22.8
410	7.1	3.2	12.9	3.9	23.7	23.2	25.4	6.4

Table 11. Flow cytometry results of family #5 (N521 & J522). Reactivities of goldfish RBCs with fluorescence-tagged antibodies are given. The letter F identifies individuals in each family. The antisera panel consists of family specific fish anti-fish (columns 1 & 2), and chicken anti-chicken (columns 3-8) antisera. The results recorded in each column are percentages of fluorescence-tagged antibodies bound by the goldfish RBCs as measured by the flow cytometer.

F	1	2	3	4	5	6	7	8
501	33.1	49.9	19.2	50.2	31.5	64.3	29.2	29.3
502	13.4	5.7	35.3	23.3	28.7	36.7	43.5	15.9
503	4.1	34.7	18.9	34.7	53.9	47.7	26.4	20.9
504	3.9	41.1	20.2	18.4	28.2	41.5	12.7	20.6
505	8.7	10.1	19.6	24.9	34.3	47.8	13.0	18.8
506	5.6	13.9	7.0	10.4	7.5	10.0	6.6	15.5
507	9.6	39.2	3.3	39.4	6.9	12.2	2.2	4.0
508	6.8	23.4	8.0	23.8	38.9	11.3	5.3	7.5
509	17.6	40.3	3.7	27.6	28.0	4.5	1.8	6.7
510	4.5	5.0	22.4	12.4	61.8	21.1	25.1	19.9
511	1.8	7.8	5.8	7.6	9.5	24.1	18.2	16.3
512	2.1	10.4	6.2	4.7	5.6	6.8	4.5	7.7
513	2.6	3.1	9.8	4.2	18.0	18.6	13.3	28.2
514	13.3	13.1	10.8	5.9	13.6	18.7	11.2	20.3
515	20.3	8.4	16.4	5.4	7.5	25.7	13.3	43.2
516	4.0	15.7	5.0	7.0	38.8	36.7	22.9	37.3
517	15.6	26.4	13.7	17.4	41.3	41.3	14.2	30.9
518	3.4	18.7	25.4	24.3	42.4	42.3	11.9	41.8
519	5.5	19.7	32.1	14.8	31.6	7.5	34.9	47.8
520	4.1	3.9	14.7	10.6	30.5	38.3	6.9	27.2

Analysis of flow cytometry results

The RBCs from individuals of four families were analyzed for positive antigen binding with the panel of antisera used in this study. Results were reported in percentages of fluorescence bound to RBCs as measured by the flow cytometer. However, no conclusive results can be reported from these data. The flow cytometer is an instrument that identifies cell populations on the basis of how those cells respond to a specific receptor molecule. In this study, we were trying to investigate the possibility of using the flow cytometer with sample sizes too small to prove adequate for serological testing by traditional agglutination assays. The results obtained by measuring fluorescence using the flow cytometer did not correlate with positive or negative agglutination results for many of the samples tested. Comparisons between the two types of tests showed negative flow cytometry results when agglutination results were clearly positive, and vice versa. There are several possible explainations for lack of concordance of agglutination results observed by traditional means and by use of the flow cytometer:

(1) It is possible that strongly positive agglutination samples were lost in the laboratory preparation of the cells for the flow cytometer. Strong agglutinations may have been discarded in the washing process necessary between addition of reagents and fluorescent antibody.

(2) Cases of positive flow cytometer results that were negative in agglutination tests may indicate the presence of additional non-MHC-related antigen receptors that were binding directly with the goat anti-rabbit fluorescent antibody.

In order for use of fluorescent-tagged antibodies and the flow cytometer to be a useful approach to testing for MHC encoded molecular variability, greater understanding of antigen receptors of the goldfish RBCs needs to be achieved.

DISCUSSION

Marker assisted selection for disease resistance

Losses to disease account for major economic losses in aquaculture. Estimates of the economic impact of diseases range from 10-15% to 30% of the cost of producing fish (Klontz 1985). Approaches to minimizing losses to disease have emphasized optimization of culture conditions, prophylactic treatments, or therapeutic agents, but have not generally considered development of disease resistant aquaculture stocks.

Variable degrees of resistance and susceptibility to infectious agents among individual fish or among stocks of fish clearly exist, yet investigation of the genetic variation underlying differential disease resistance has not been undertaken. Demonstration of genetic variability and linkage of marker alleles to different disease resistance traits are necessary steps for carrying out selection for improved disease resistance in aquaculture stocks (Hallerman and Beckmann 1988). Development of a suitable collection of genetic markers would be a major contribution toward achievement of such goals. This study demonstrated the utility of hemagglutination assays based on the use of chicken-derived antisera in development of suitable genetic markers. The utility of fish anti-fish reagents was also suggested.

Upon development of a suitable collection of MHC markers, individuals, families and populations both resistant and susceptible to particular diseases could be screened for possession of given markers or marker haplotypes. For example, serological-level markers in the MHC complex have been found for bovine leukemia virus (Lewin and Bernoco 1986), bovine mastitis (Larsen et al. 1985, Oddgeirsson et al. 1988, Spooner et al. 1988), bovine ocular squamous cell carcinoma and enzootic bovine leucosis (Stear 1988), bovine theileriosis (Morrison et al. 1988), equine sarcoid infection and

caprine virus-induced arthritis (Ruff and Lazary 1988), and Marek's disease of poultry (Hansen et al. 1967, Longenecker et al. 1976, Briles et al. 1977, 1983). Observed relationships between alleles of the chicken B system and the degree of resistance to Marek's disease (Briles et al. 1977) has been attributed to differences in immune response, either through histocompatibility-linked Ir genes or through antigen recognition phenomena controlled by the B complex itself.

Knowledge of resistance and susceptibility markers can be applied in marker-assisted selection for improved immune responsiveness and disease resistance, as has been demonstrated for Marek's disease in chicken and scours in pig (Gavora and Spencer 1983).

Once a direct or linkage effect of a polymorphism upon a trait of economic importance has been determined, a few generations of selection will suffice to bring it to high frequencies (Beckman and Soler 1987). In addition, a genetic marker associated with a direct effect can be readily introgressed from one population to another through appropriate crosses (Beckman and Soller 1987). The expense of research into establishing and utilizing genetic markers would thus be returned through the economic benefits attributed to reduced losses to disease.

Utility of respective serological approaches for developing antisera

The purpose of this research was to develop a practical method for identifying putative MHC haplotypes in fishes. The data exhibited the inheritance of RBC surface antigens within and between families and also the basis for utilizing reagents specific to the chicken B MHC system to identify putative MHC haplotypes in fishes. Futher, the results of this study suggest the characterization of specific putative MHC haplotypes in goldfish.

It is not inequivical that molecules "seen" by reagents used were indeed MHC. Immunoprecipitations should have been done to see if anti-fish antibodies pulled down proteins with

a molecular reaction equivalent to other known MHC molecules present on RBCs of chickens or other species. When making alloantibodies there are a lot of different types of proteins that can be involved. Immunoprecipitation should have been determined before and after absorptions.

Widespread adoption of a serological approach to marker-based improvement of disease resistance in aquaculture stocks will depend on demonstration of the sensitivity and cost-effectiveness of marker-based methods. In this research, fish anti-fish, chicken anti-chicken, and chicken anti-fish antisera were utilized in order to evaluate the sensitivity of serological and flow cytometry procedures in identifying MHC markers on the surface of fish RBCs.

Preliminary data collected with the fish anti-fish antisera in agglutination assays suggested that the antisera could be developed for identifying inheritance of RBC surface antigens. With immunizations between broodstock and progeny, antisera could be produced that would aid in the identification of specific MHC haplotypes in fish species. A process similar to that used in chicken (Briles 1960, 1983) should yield reagents of similar nature for testing for MHC-related variability in fish. Production of the antisera would involve propagation of several generations of fish families and development of a complete collection of antisera. This process would involve an experiment of greater scope than this study. However, the results of my study suggest that the development of fish anti-fish antisera could be successful.

Reactivities observed with use of the chicken anti-chicken antisera showed it is likely to be possible to identify piscine MHC haplotypes using chicken-derived reagents. Many different agglutination patterns were observed with the chicken anti-chicken antisera, and there were phenotypes that were observed within and between families. Differences in patterns of agglutination indicate that antibody-antigen recognition with the chicken anti-chicken antisera and fish antigens occurred. More data on reactivity of specific chicken anti-chicken antisera and fish RBCs needs to be collected and analyzed. Collections of MHC haplotype-specific chicken anti-chicken antisera

already exist. However, access to such chicken anti-chicken antisera is somewhat limited, as they are not commercially distributed.

The production of the chicken anti-fish antisera did not prove useful in this study. The positive results observed in all but one individual could have been due to non-specific binding not eliminated during the absorption process, or by recognition of cellular antigens to antibodies produced by the chicken B system. Use of chickens of known MHC genotypes for production of chicken anti-fish reagents for classifying piscine agglutination phenotypes would require cooperation with those who have characterized the chicken MHC, complicating the process of characterizing the piscine MHC.

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VITA

Gail Diane Maxey was born April 6, 1955 in Roanoke, Virginia to Sam and Dawne Maxey. She attended school in the Roanoke area, graduating from William Byrd High School in 1973. She was married in 1973, and had one son, Brian, in 1975. In 1978 she graduated from the Lewis-Gale School for Certified Laboratory Assistants, and worked in the medical lab field for 10 years. In 1987, she enrolled in Hollins College to complete a bachelors degree. While at Hollins College, she became actively involved with the Roanoke Valley Governor's School for Science and Technology, and with the Science Museum of Western Virginia. She graduated from Hollins College in May 1990, with a BA in biology and also completed the Virginia state requirements for teaching secondary biology. She moved to Blacksburg, VA in May of 1990, and worked as a technician in the aquaculture program at Virginia Polytechnic Institute and State University (VPI & SU). In the fall of 1990, she enrolled as a student in the Department of Fisheries and Wildlife Science at VPI & SU and began working towards a M.S. degree in immunogenetics.