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APPLICATIONS OF MONOCLONAL ANTIBODIES IN AQUACULTURE

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ABSTRACT

Monoclonal antibodies (MAbs) currently are being applied to the study of fish immunology and fish infectious diseases. MAbs to fish immunoglobulins (Igs) have helped isolate fish Igs, identify heavy and light chain variants in fish Ig, study the ontogeny of B lymphocytes, and improve techniques for the measurement of fish Ig and specific antibodies (Abs). MAbs have been obtained against several leucocyte surface antigens and are being used as markers for different subsets of fish leukocytes: neutrophils, non-specific cytotoxic cells and cells responsible for the mixed leucocyte reaction. The sensitivity and specificity of many immunoassays for identifying fish pathogens have been improved by the use of MAbs. Further improvement of these tests is currently being attempted by the use of MAbs together with the polymerase chain reaction (PCR). Epizootiological studies of fish diseases are begining to emerge from the use of these reagents and techniques. In the near future these new methods should detect low levels of pathogens in adult carriers and perhaps in eggs, thus helping to prevent the dissemination of fish diseases. MAbs to fish pathogens are also being used for passive immunization studies as well as for conformational probes in the development of genetically engineered vaccines.

Key words: monoclonal antibodies, aquaculture, immunoglobulins, leucocyte markers, diagnosis, vaccines.

INTRODUCTION

Since the first report by Kohler and Milstein in 1975 on the generation of hybridomas by fusion of B lymphocytes with myeloma cells,the production of virtually unlimited quantities of antibodies (Abs) of a single specificity has been possible. The availability of these monoclonal antibodies (MAbs) has had a great impact on basic research areas (such as immunology, microbiology and cell biology) and has found many different applications in human and veterinary medicine.

Although aquaculture for human comsumption also includes molluscs and crustaceans, most applications for monoclonal antibodies (MAbs) in aquaculture have been with teleost fish. The rainbow trout (*Oncorhynchus mykiss*, W), the salmon (*Oncorhynchus* sp and *Salmo* sp), the carp (*Cyprinus carpio*, L), the catfish (*Ictalurus punctatus*) and a few marine species like turbot (*Scophtalmus maximus*, L) are the cultured teleosts on which MAbs have been used most frequently. In this review we will focus on applications of MAbs in two main areas: one is studying the humoral and cellular immune response of the host, and the other is characterizing the pathogen (virus, bacteria or parasite), either for the improvement of diagnostic techniques or for the identification and characterization of the antigenic determinants involved in protection in order to define potential candidates for the development of recombinant vaccines.

FISH LEUCOCYTE MARKERS

The problem and its difficulties

One of the most effective approaches for identifying and separating the different cell lineages of the lymphohemopoietic system of vertebrates is the analysis of cell surface glycoproteins. Depending on the cell lineage and on its differentiation or activation stage, a cell expresses characteristic or unique surface antigens. Since the advent of hybridoma technology, large collections of MAbs have been developed against leukocytes of humans, mice and species of agricultural interest, such as the cow, pig, sheep, horse, and chicken. These have been extremely useful in the study of normal and diseased conditions.

Until the introduction of MAb technology, the major problem in studying cell surface molecules was to produce a specific Ab against individual cell surface molecules. Since all cell surface molecules might be potentially antigenic, unless a pure antigen was used, xenogenic immunization would result in polyclonal antisera containing a mixture of many different Abs with specificities for unique as well as for shared antigens, with the consequent lack of specificity (cross-reactivity with common antigens present in different types of cells). Even in the case where a pure antigen was used, a mixture of Abs against different determinants (which could also be shared by related proteins) and with different affinities would result. Several approaches have been attempted to reduce the unwanted crossreactivity of the polyclonal antisera, such as absorption with antigens from other cell lines. However no general solution has been obtained.

An example of this crossreactivity was the staining of fish thymocytes by polyclonal antisera to serum immunoglobulins (Ig). Those results were interpreted as the presence of sIg+ thymocytes (i.e. putative B cells) in several species of fish (Emmrich *et al.*, 1975; Warr *et al.*, 1976; Clem *et al.*, 1977) until some evidence was obtained suggesting that it was an artifact caused by the crossreactivity of the polyclonal antisera (PAbs) with carbohydrate moieties on a variety of non-Ig molecules (Yamaga *et al.*, 1978a,b). Later analysis with anti-Ig MAbs supports the notion that fish thymus contains few, if any, B cells (De Luca *et al.*, 1983).

Strategies used

Several studies indicate the existence in fish of a lymphocyte heterogeneity similar to that seen in higher vertebrates. In this regard, fish lymphocytes could be separated, according to the expression of surface Ig (sIg) into subpopulations representing B cells and T (and null cells) cells based on their respective functional activities in "*in vitro*" assays; for example, a) proliferative responses to mammalian B and T cell mitogens such as LPS or ConA (De Luca *et al.*, 1983; Sizemore *et al.*, 1984), b) Ab responses to thymus dependent and thymus independent antigens (Miller *et al.*, 1985; Miller *et al.*, 1987), and c) mixed lymphocyte reactions (Miller *et al.*, 1986). However, in contrast to mammals and birds where large collections of MAbs have been developed against their leucocytes, the number of MAb recognizing specific membrane markers of fish leucocytes is still scarce. The identification of Ig-bearing lymphocytes (B cells) is not a problem. For instance, MAbs against serum Ig have been reported for several teleost species (see Table 1). So far, only a few MAbs recognizing Ig negative subpopulations have been reported, which underlines the scarcity of T lymphocyte markers in fish.

TABLE 1: Some fish species for which MAbs against serum Ig are available.

Species (latin name)	References
Carp (*Cyprinus carpio*)	Secombes *et al.*, 1983.
Channel catfish (*Ictalurus puntactus*)	Lobb & Clem, 1982; Lobb *et al.*, 1984, 1988.
Atlantic salmon (*Salmo salar*)	Killie *et al.*, 1991.
Turbot (*Scopthalmus maximus*)	Estevez *et al.*, 1994a.
Atlantic cod (*Gadus morhua*)	Israelsson *et al.*, 1991.
Rainbow trout (*Oncorhynchus mykiss*)	De Luca, 1983; Thuvander *et al.*, 1990; Sánchez, 1991; Sánchez *et al.*, 1993b.

Inasmuch as the thymus is considered the organ where T cell precursors mature, thymocytes have been used as the immunogen for the development of MAbs against T cell markers. One of the first to apply this strategy to fish was Secombes *et al.* (1983) who developed

MAbs against carp thymocytes. However, most of the MAbs obtained were also positive for carp Ig by ELISA. One of these MAbs, the WCT23, was further characterized by Van Diepen *et al.* (1991). WCT23 stained the vast majority of lymphocytes from carp pronephros as well as all the granulocytes, but did not react with erythrocytes. By Western blotting it reacted with the 70 Kd heavy chain of serum Ig and many other molecules in the lymphocyte membrane preparations. The authors conclude that this MAb probably recognises a common antigenic determinant present on several membrane and serum molecules, including Ig. PAbs and MAbs have also been raised against either thymocyte isolated membranes (Chylmonczyk, 1993, personal communication) or thymocyte isolated membrane proteins (Tournefier *et al.*, 1991).

Other strategies include the purification of different cellular subsets to be used as immunogens for producing MAbs. Several examples will illustrate this method. For instance, a MAb (13C10) reacting with catfish peripheral blood lymphocytes was obtained by using peripheral blood leucocytes as immunogen. This MAb reacted with cells which provide T-helper activity and which only in the presence of accesory cells responded to ConA (Miller *et al.*, 1987). By flow cytometry more than 95% of the thymocytes were recognized by the MAb 13C10. However, it also recognized some thrombocytes. This MAb binds relatively high molecular weight antigens on catfish cells that were considered to be analogous to Thy-2, and is being used as a pan catfish anti-T cell like reagent.

Evans *et al.* (1988) developed MAbs against a population of cells from catfish purified by flow cytometry called by analogy to mammalian NK cells, nonspecific cytotoxic cells (NCC). Two MAbs were able to inhibit the cytolysis of several target cell lines (NC-37, MOLT-4, K562, P815, U937, etc) by the NCC. These MAbs recognized a dimer of 41 Kd and 38 Kd polypeptides present in the cell membrane and stained approximately 23-39% of catfish anterior kidney cells, 40-54% of catfish spleen cells and 1.6-2.5% of catfish peripheral blood cells. Interestingly the determinants recognized by these MAbs seem evolutionarily conserved because these MAbs were able to inhibit human and rat NK cytotoxicity (Harris *et al.*, 1991).

Other examples of MAbs to fish leucocytes are relatively recent. Greenlee and Ristow (1993) have also obtained a MAb which suppresses the cytolysis by trout NCC. Their MAb suppresses the *in vitro* mixed leucocyte reaction by reacting with the N-linked oligosaccharides broadly expressed on trout leucocytes. A MAb (13C5) that reacts specifically with catfish neutrophils based upon histochemical and functional criteria has been described by Bly *et al.* (1990). Cells stained by MAb 13C5 show positive reactions with Sudan Black B and nitroblue tetrazolium (NBT) and are phagocytic. This MAb has been used to study stress-induced neutrophilia in this fish. Devold Maaseide *et al.* (1993) have developed a MAb that reacts selectively with salmon granulocytes and monocytes in peripheral blood. Finally, Slierendrecht *et al.* (1993) developed a panel of MAbs against trout leucocytes by immunizing with a cell preparation depleted of Ig-bearing cells. One of their MAbs seems specific for granulocytes and thrombocytes as revealed by flow cytometry and electron microscopy.

Overall, from the little sucess that has been obtained to date, it seems clear that new strategies and/or more effort(s) are needed in order to obtain the necessary fish leucocyte markers. This will remain for some time one of the most anticipated developments in fish immunology.

FISH IMMUNOGLOBULINS (Igs)

Structural studies of fish Igs

The major serum Ig found in fish is a molecule that resembles the mammalian IgM. It consists of 70 KDa heavy chains (μ or H) and 22-25 KDa light chains (L) in equimolar amounts. Fish IgM-like has been isolated from many species of fish, both teleosts and elasmobranchs. In teleosts, the Ig is composed of 4 subunits, each subunit comprising 2 covalently linked H-L chain pairs, whereas in the elasmobranchs Ig is a pentamer composed of 5 subunits (reviewed by Sánchez and Coll, 1989 and by Wilson and Warr, 1992).

Previous studies reported the existence of structural heterogeneity and suggested an antigenic heterogeneity in teleost Igs. For instance, L chain diversity is detectable by electrophoresis in catfish (Lobb *et al.*, 1984) and trout (Sánchez *et al.*, 1989). Although these results were consistent with the presence of isotypic variants in fish, these variants were not firmly established until the development of MAbs. With MAbs, structural and antigenic variants have been demonstrated in catfish (Lobb *et al.*, 1984) and trout (Sánchez and Dominguez, 1991) L chains of serum Ig. The existence of antigenic variants of H chains also has been demonstrated in catfish serum Ig (Lobb and Olson, 1988) and suggested for salmon serum Ig (Killie *et al.*, 1991).

Catfish Ig contains two different classes of L chains defined by MAbs 3F12 and 1G7. These F and G L chains are present in all catfish and account for all the serum Ig L chain. They show differences by peptide mapping and in the N-terminal sequence. Approximately 60% of the serum Ig contains F chains and the other 40% contains G chains. Lobb and Olson (1988) also developed MAbs (3D11, 3E11 and 1H6) that identified structurally different H chains with each variant representing about 20% of the serum Ig. There should be at least one additional H chain type not yet defined by a MAb in order to account for the remaining 40%. These variants seem to be differentially expressed during an immune response. For example, after long-term immunization of catfish with DNP, 90% of the anti-DNP Abs contained F L chains and 50-70% of these Abs contained H chains reactive with MAb 3D11.

In trout, 2 antigenically and structurally different L chains have been defined using MAbs (Sánchez and Domínguez, 1991). MAb 2H9 reacts with the L chains of 26 KDa, whereas MAb 2A1 reacts with the L chains of 24 KDa. These L chains showed peptide differences after V8 protease digestion. The two Ig populations defined by these MAbs were present in every trout sera tested. However, both Ig populations only acounted for 33% of the serum Ig, suggesting a higher degree of L chain heterogeneity in trout than that reported in catfish. There should be at least one more L chain variant, in the remaining 67% of Igs not recognized by any of the 2 MAbs (2H9 and 2A1).

Although the above catfish and trout serum Ig variants were present in every fish serum tested, it is not yet resolved if the differences lie in the constant or in the variable regions of the chains, and therefore, if they are true isotypes or subgroups (for instance, consensus sequences in the framework regions, shared by the members of a variable gene family). This issue will probably be resolved when more data from ongoing studies at the molecular genetic level become available.

Anti-Ig MAbs have also been used to study the differentiation of B lymphocytes during fish ontogeny. Using MAbs specific for H chains of rainbow trout Ig, Castillo *et al.* (1993) investigated the appearance of cells bearing cytoplasmic or sIg at different stages of development. Lymphocytes showing cytoplasmic μ chains were detected as early as 12 days before hatch, whereas sIg+ cells were first detected at day 8 before hatch. In a similar way, during the development of mammalian B cells, the rearrangement and expression of H genes precedes that of the L genes, and thus in the pre-B cell, the μ chains but not L chains can be detected in the cytoplasm. The recent availability of MAbs to L chains of trout Ig (Sánchez *et al.*, 1993) will allow further studies on the ontogeny of B cells in this fish species.

Determination of total Ig levels

Determination of fish serum Ig levels has been used as a general parameter to assess immunocompetence in vaccination programs or during disease, and also to evaluate possible effects of environmental conditions on the immunological status of a fish population. This was usually done by single radial immunodiffusion, using PAbs (Ingram and Alexander, 1979; Voss *et al.*, 1980; Olesen and Vestergard-Jorgensen, 1986; Havarstein *et al.* 1988; Fuda *et al.*, 1989). However, this assay is of low sensitivity and takes a long time. The development of MAbs to the Ig of some commercially important species, and therefore their unlimited availability, has lead to the development of ELISA assays of higher specificity, sensitivity and reproducibility and easier standardization. In addition, ELISA assays allow the determination of Ig concentrations in a large number of samples in a short time and using small volumes of sera (or secretions). However, when using MAbs for this purpose, one must be sure that the MAbs recognize all the molecules of Ig and not a particular isotype or variant.

ELISAs for quantification of Ig using anti-H MAbs have been described for trout and other salmonids (Thuvander *et al.*, 1990; Sánchez *et al.*, 1993a), for catfish (Klesius, 1990) and for turbot (Estevez *et al.*, 1994a,b). Since at least some of the determinants recognized by these MAbs seem to be conserved in the Ig of some related fish species, these assays may also be useful for quantitation of Ig of those species. Thus, the MAbs used to quantify trout Ig by ELISA cross-react with Igs of other salmonid species, like Coho salmon, sockeye salmon, chum salmon, etc.(Sánchez *et al.*, 1993b).

MAbs of high titre have been also used to design simpler methods for fish Ig purification based on affinity chromatography (Sánchez *et al.*, 1990).

Determination of pathogen-specific Ig levels

An approach to demonstrating prior exposure or latent host infection by a pathogen is the detection of specific Abs to its antigens in the serum of the host. MAbs have also been successfully applied in ELISA to determine fish serum Abs levels following survival of infection or vaccination with viruses (rhabdoviruses, viral haemorrhagic septicaemia, VHSV, and infectious haematopoietic necrosis (IHNV) and bacteria (*Renibacterium* and *Vibrio*).

A VHSV capture ELISA using MAbs has been described to measure VHSV specific trout Ig. This assay proved to be more sensitive and less time- and material-consuming than either immunofluorescence or neutralization (Olesen *et al.*, 1991). A simplified ELISA assay was also used by Ristow *et al.* (1993) to detect Abs to IHNV. Sera from 143 trout surviving IHNV infection were analyzed for specific Ab against the virus by ELISA, plaque neutralization test (PNT) and blotting. The majority of the sera tested demonstrated positive anti-IHNV Ab titers in the ELISA (92%) and the PNT (82%), unlike a previous study carried out by Jorgensen *et al.* (1991) where only 45% of the fish tested exhibited positive PNT and ELISA titers. However, some of the fish used for Ristow *et al.* (1993) were exposed to IHNV 5 times and not 1-2 times as in Jorgensen *et al.* (1991). Fish serum with high anti-IHNV Ab titers detected by PNT or ELISA did not necessarily have a positive blot to IHNV structural proteins. The protein most frequently recognized by the fish sera was the viral glycoprotein G, which has been shown to induce protection against IHNV infection in different salmonid species (Engelking and Leong, 1989).

The Ab response to bacteria is usually monitored by using agglutinin and precipitin methods. However, the results obtained with these tests are frequently complicated by non-Ab-mediated precipitates induced by C-reactive protein (Ellis, 1985), by the need of relatively high Ab concentrations and by the tendency of some bacteria (*R. salmoninarum*) to autoagglutinate. ELISAs with MAbs do not have the above mentioned problems and in addition provide the advantages of greater sensitivity and large-scale testing. As a consequence, they are slowly replacing the above mentioned methods. Thus, Estevez *et al.* (1994c) found a MAb based ELISA to be a valuable tool for the monitoring the low Ab responses in turbot, such as that occurring following immersion vaccination to *Vibrio anguillarum*, which were undetectable by agglutination tests.

As mentioned above, anti-Ig MAbs have also been used in western blotting techniques to identify the antigens recognized by the fish. Bartholomew *et al.* (1991) compared the specificity of the Ab response to *R. salmoninarum* in naturally infected salmon to the response in immunized salmon. Most of the Abs were directed against a 58 kDa protein complex. In addition, Ab from the immunized salmon recognized 4 other *Renibacterium* proteins with lower molecular masses. A similar approach was used to determine the antigen specificity of turbot Ab induced by infestation with the parasite *Tetramicra brevifilum* (Leiro *et al.*, 1993).

A different and ingenious modification of the sandwich ELISA for Ig quantitation is the ELISPOT that allows the enumeration of the Ab secreting cells. Siwicki and Dunier (1993) developed an ELISPOT for quantification of total and specific Ab secreting cells in spleen, head kidney and blood of rainbow trout immunized with a *Yersinia* vaccine.

DIAGNOSTIC METHODS FOR FISH PATHOGENS

Diseases of teleost fish and their diagnosis

Rapid detection and identification of infectious outbreaks is important for better management of diseases and to avoid the dissemination of pathogens. This is especially important in the case of fish viruses because vaccines or antiviral drugs are not available for the control of these diseases and avoidance of the pathogen is the only means of control. The major mortality-causing diseases in aquaculture of fish are caused by viruses and to a lesser extent by bacteria and parasites. Infectious pancreatic necrosis (IPN), viral haemorrhagic septicaemia (VHS), infectious haematopoietic necrosis (IHN), spring viremia of carp (SVC) and channel catfish virus (CCV) disease are among the major diseases causing severe mortalities of farm-reared fish.

TABLE 2: Important fish pathogens for which MAbs have been developed

Pathogen	References
Viruses	
Infectious pancreatic necrosis (IPN)	Caswell-Reno *et al.*, 1986; Wolski *et al.*, 1986; Christie *et al.*, 1990; Dominguez *et al.*, 1990; Tarrab *et al.*, 1993; Lecomete *et al.*, 1992.
Viral hemorrhagic septicemia (VHS)	Mourton *et al.*, 1990; Sanz *et al.*, 1992; Lorenzen *et al.*, 1988,1990; Sanz *et al.*,1992.
Infectious hematopoietic necrosis (IHN)	Shultz *et al.*, 1985, 1989; Winton *et al.*, 1988; Ristow & Arnzen, 1989,1991.
Channel catfish (CCV)	Arkush *et al.*, 1992.
Eel virus (EVE)	Chi *et al.*, 1991.
Bacteria	
Renibacterium salmoninarum	Wiens & Kaattari, 1991.
Aeromonas salmonicida	Rockey *et al.*, 1991.
Vibrio sp	Svendsen & Larson, 1988; Espelid *et al.*,1988.
Parasites	
Ceratomyxa shasta	Bartolomew *et al.*, 1989.
Ichthyophthirius m.	Dickerson *et al.*, 1986; Lin & Dickerson, 1992.

Current standard methods for the isolation and identification of fish viruses require the use of established fish cell lines and serum neutralization tests. Although PAbs have been used to detect fish pathogen antigens, their titer and specificity (anti-host cell activity) have limited their wide-scale use (Winton *et al.*, 1988; Lecomte *et al.*, 1992), and therefore MAbs are rapidly replacing them (Coll, 1993).

MAbs are already available to most of the pathogenic microorganisms causative of the economically more important aquaculture diseases (see Table 2). Fast, simple, specific and sensitive techniques for diagnosis of clinical cases of these diseases have been developed based on the use of MAbs.

Application of MAbs to the identification of pathogen proteins

Neutralization. Only a few neutralizing MAbs have been used in clinical fish diagnosis (Hattenberger *et al.*, 1989; Eaton *et al.*, 1991) because neutralizing MAbs with a high enough titre have been difficult to obtain for IPNV (Dominguez *et al.*, 1990, 1991), VHSV (Lorenzen *et al.*, 1990, Sanz and Coll, 1992b) and IHNV (Winton, 1991; Winton *et al.*, 1988; Ristow and Arnzen, 1991). Arkush *et al.* (1992) developed MAbs against an isolate of catfish virus (CCV). They selected 3 MAbs for indirect fluorescent antibody tests (IFATs) and 4 neutralizing MAbs. These MAbs were tested for their binding and neutralization activities against 4 different isolates of CCV.

Immunofluorescence and immunoperoxidase. Although the use of MAbs increases the specificity and reproducibility of these methods, it remains to be seen whether or not the use of MAbs will increase the sensitivity of these techniques (Sanz and Coll, 1992c,d; Arnzen *et al.*, 1991; Ristow and Arnzen, 1991). Two anti-IHNV MAbs were selected that exhibited good immunofluorescence with no background fluorescence. These MAbs reacted with all strains of IHNV tested, which included the different electropherotypes identified. The FAT (fluorescent Ab test) has been used for the detection of IHNV from organs of infected juveniles and adult carriers. IHNV was detected after 48 h in cell lines inoculated with as low as $10^{2.5}$ pfu/ml of fish tissue (LaPatra *et al.*, 1989). Cytoplasmic fluorescence in the form of a cap was visible at 6-8 h with both anti-glycoprotein and anti-nucleoprotein MAbs. Full cytoplasmic fluorescence was present at 12-16 h (Arnzen *et al.*, 1991). Although fluorescent techniques are a rapid means of detecting the presence of IHNV in cell culture, for the complete characterization of any IHNV isolate, neutralization studies performed with PAbs or MAbs to the glycoprotein are actually required.

Using a MAb specific for VP2, Babin *et al.* (1991b) were able to detect IPNV in CHSE-214 cells infected with a m.o.i. of 0.01, as early as 3 h post-infection. Clear cytoplasmic fluorescence was observed at 6-9 h.p.i. Of sixteen MAbs tested, two were specific for the Sp strain and one reacted selectively with the Ab, Te and Canada 1 strains.

MAbs also have been used for detection of *Vibrio* (V.) by ELISA and immunofluorescence on tissue smears (Espelid *et al.*, 1988). Antigens of *V. salmonicida* were also specifically identified with immunohistochemical techniques in formalin-fixed, paraffin-embedded tissue specimens from heart, liver, spleen, kidney, and gut from Atlantic salmon suffering from experimentally induced cold water vibriosis (Evensen *et al.* 1991).

Bartholomew *et al.* (1989) developed a panel of MAbs against the myxosporean *Ceratomyxa shasta*. The MAbs reacted with trophozoite and sporoblasts stages but did not react with *Ceratomyxa* spores. One hybridoma produced Abs of high specificity for *Ceratomyxa* pre-spore stages. This MAb was effective in detecting low numbers of *Ceratomyxa* both in histological sections and in smears of intestinal material from infected fish, making it suitable for use as a diagnostic reagent, either in immunofluorescence or in immunohistochemistry.

A panel of 34 hybridomas, secreting MAbs reactive with an infective stage of *Ichthyophthirius multifiliis*, one of the most pathogenic protozoan parasites of freshwater fish, was obtained by Dickerson *et al.* (1986). Three of these MAbs were cloned and tested for ELISA and immunofluorescence assays.

Agglutination. Agglutination using MAbs or the highly reproducible latex techniques (Coll, 1993) has not yet been widely applied to pathogens in aquaculture. This could be an area of further application.

Enzyme immunoassay, ELISA. Several ELISA systems based on MAbs to fish pathogens have been described for viruses and for some bacterial diseases. We will review some of them.

Domínguez *et al.* (1990) described the use of MAbs with detection levels of 10 ng of purified virus/ml to detect IPNV. Acutely infected fish might be detected by this technique but its sensitivity is not yet high enough to detect adult carriers. Rapid serotyping of IPNV by ELISA has also been recently described with serotype specific MAbs (Domínguez *et al.*, 1991).

Sensitivity is also a problem in the detection of fish rhabdoviruses. A sensitivity of 1 ng/ml or 0.2 ng/ml has been described for VHSV by using serotype specific MAbs against the G protein (Mourton *et al.* , 1990) or for all serotypes of VHSV by using MAbs against the N proteins (Basurco *et al.*, 1991; Sanz and Coll, 1992a; Mourton *et al.*, 1992). The use of anti-N protein MAbs of high titre allowed the recognition of all the serotypes of VHSV whereas the use of high-ionic strength buffers that disrupted the nucleocapsids increased the sensitivity about 100-fold (Sanz and Coll, 1992a). The use of two non-overlapping MAbs has simplified the procedure to a one-step assay which unexpectedly added a further 10-fold increase in the sensitivity. The high sensitivity obtained correctly detected fingerling trout (Sanz and Coll, 1992a) or turbot (Braña *et al.*, 1994) infected in the laboratory.

Despite the high numbers of MAbs developed against the G or the N proteins of IHNV (Ristow and Arnzen, 1989, 1991), the present state of ELISA to detect IHNV has lower sensitivity

than for VHSV (S. Ristow, personal comunication). It is obvious that preparation of an ELISA for all the IHNV types isolated in the United States and Europe will take a lot more work and PCR would probably be easier and more sensitive. Similar assays for SVCV have not yet been possible with MAbs (Rodak *et al.*, 1993).

Renibacterium salmoninarum causes a chronic disease of salmonid fish known as bacterial kidney disease (BKD). The reliability of anti-Renibacterium PAbs for the diagnosis of BKD has been questioned because of cross-reactivity problems (Austin *et al.*, 1985). Structural, antigenic and biological characterization of the Renibacterium 57 KDa antigen has been studied with MAbs (Wiens and Kaattari, 1991) and a MAb-based ELISA was developed for the diagnosis of BKD (Hsu *et al.*, 1991). This ELISA was more sensitive than fluorescence for the diagnosis of BKD in asymptomatic fish and could detect Renibacterium at 0.05-0.1 μg/ml.

One of the obvious goals in this area is to establish a "multivalent" reagent kit which can be used in a simple procedure to screen for the presence of pathogenic microorganisms causing similar clinical illnesses. For instance, McAllister and Schill (1986) have described an immunodot assay using PAbs for identification of the three more frequent viral infections of salmonids (IPN, VHS and IHN). Ideally, a panel of different ELISA systems based on MAbs (for instance, IPNV, VHSV, SVCV and IHNV) could be used to provide both rapid detection and identification during disease.

Immunoblot and Immunodot. MAbs have been used in immunodot tests for antigenic characterization and improved diagnosis of IPNV (Lilipun *et al.*, 1989; Caswell-Reno *et al.*, 1989; Babin *et al.*, 1990, 1991a). Schultz *et al.* (1989) developed an immunodot assay for detection of IHNV by using MAbs that detected 10^2 $TCID_{50}$. During the last years, VHSV has appeared in North America and IHNV has appeared in Europe. Therefore, methods to distinguish both rhabdoviruses are being developed, some of them based on MAbs (Ristow *et al.*, 1991). Ristow *et al.* (1991) developed an immunodot assay with 2 MAbs that recognize conserved epitopes on the nucleoproteins of VSHV and IHNV. MAb 1NDW14D identified 80 of the 81 IHNV isolates spotted onto the nitrocellulose, but none of the 8 VHSV isolates. MAb IP5B11 stained all 8 samples of VSHV but none of the IHNV isolates. Neither MAb crossreacted with other rhabdoviruses like SVCV, pike fry rhabdovirus, rhabdovirus anguilla or Danish eicosid rhabdovirus.

A biotinylated MAb was used in an immunoblot assay able to detect 10^4 pfu/ml of IHNV in cell culture fluid, but was not suitable for detecting IHNV in homogenates from fish organs because false positive reactions resulted from the binding of MAb to some of the organ proteins (Schultz *et al.*, 1989). A modification of this assay that avoids the interference of the organ homogenates has been reported by Babin *et al.* (1991). Instead of the direct binding of the cell culture supernantant or organ homogenate to the nitrocellulose membrane, these authors use a MAb to capture the virus. The bound virus is then detected with a second biotinylated MAb.

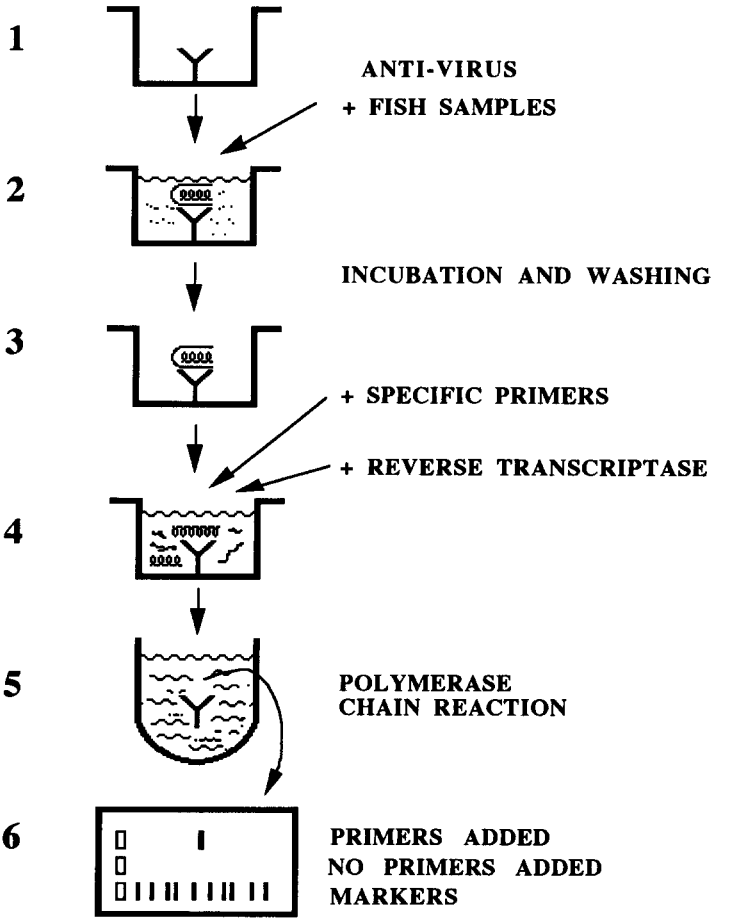

Figure 1. Scheme of ELISA - PCR. 1. Wells of a 96-well plate are coated with anti-virus MAb of high affinity. 2. Homogenates from the kidney of diseased fish are incubated in the wells to capture the virus. 3. Wells are washed to eliminate potentially interfering fish debris. 4. Addition of virus-specific primers and reverse transcriptase to convert viral RNA to DNA 5..Transfer to an eppendorf tube where polymerase chain reaction amplifies a small and conserved DNA fragment (300-500 bases). 6. Analysis of specific amplified bands by gel electrophoresis.

ELISA - PCR. New developments are expected with this technique due to the possibility of first capturing the virus with a high affinity MAb in solid-phase and later amplifying short stretches of the captured viral genome by the polymerase chain reaction (PCR) technique (Fig. 1). The viral DNA amplification can take place in a few hours and in addition it can be made highly specific to the virus by selecting sequence specific primers (Sobrino *et al.*, 1989). Preliminary results (Estepa

et al., unpublished) show that this technique could be applied to cell culture supernatants infected by VHSV and IPNV (McAllister *et al.*, 1991).

Cytofluorometry. Very recently this technique was reported for detection of virus infected cells by using PAbs (Rodriguez *et al.*, 1991). Due to the increase in specificity and sensitivity, this technique should find more use in diagnosis, although the high cost of the equipment represents a serious limitation for its broad use. Once MAbs are available, this technique should help basic studies on antigen expression as possible targets for ADCC (antibody-dependent cellular cytotoxicity) or complement- mediated lysis of virus infected cells. This was the case when MAbs were used for the first time to detect viral epitopes in the membrane of infected fish macrophages (Estepa *et al.*, 1992,a,b) or fish stroma (Diago *et al.*, 1993). Not only neutralizing epitope(s) of the glycoprotein G of VHSV were expressed in the membrane of infected trout macrophages but also epitope(s) of the nucleoprotein N.

EPIZOOTIOLOGICAL STUDIES

Panels of MAbs that recognize common, serotype- and isolate-specific epitopes of a virus or bacteria have been used to analyse the geographic distribution of different fish pathogen types, to determine the relatedness of pathogens associated with disease outbreaks, and for tracking the movement of a particular pathogen through fish stocks. Some examples are discussed.

Tarrab *et al.* (1993) analysed a collection of IPNV isolates with a panel of MAbs produced against three strains of IPNV (HF, LW, JA). All IPNV strains seemed closely related when examined with MAbs anti-VP3 but were classified differently with anti-VP2 LW and JA MAbs. North American strains showed neutralization epitopes different from the European ones. From these observations, these authors speculate that IPNV strains acquire new neutralization epitope motifs when propagated in different geographical environments. Other similar studies have been conducted with IPNV isolated in Norway (Christie *et al.*, 1990).

A similar analysis of the antigenic relatedness of the 3 European IHNV isolates to representative strains from North America was performed using neutralizing MAbs. The RB/B5 and the 193-110/B4 MAbs neutralized all the European isolates. However, partial reactivity of the virus isolates from France with the SRCV/A4 MAb distinguished them from the virus recovered from salmonids in Italy (Arkush *et al.*, 1989). Winton *et al.* (1988) analysed 12 IHNV isolates obtained in different geographic areas by neutralization with MAbs developed against 3 strains of IHNV. The MAbs recognized antigenic variants among the isolates and could be used to separate the viruses into 4 groups. The members of each group tended to be related by geographic area rather than by source, host species, virulence, or date of isolation. Using the same panel of neutralizing MAbs, Eaton (1991) analysed the reactivity of 9 Alaskan isolates of IHNV from 3 different species of salmonids. The viruses could be divided into 3 subgroups based on the ability or inability to react with the 4 MAbs.

By using a panel of 27 MAbs and by sero-neutralization with 6 anti-glycoprotein MAbs, Ristow and Arnzen (1991) analysed a collection of 17 isolates of IHNV from different geographical regions, years and stocks. Wide antigenic differences were found in the nucleoproteins and the glycoproteins of the isolates, corresponding to the different electropherotypes. Serum neutralization with 2 of the anti-glycoprotein MAbs divided the 17 isolates into those which were neutralized with both MAbs and those which were not. The results from these studies could have important implications for vaccine development, if it is demonstrated that the epitopes identified by these MAbs are important in protection. Although the ideal candidate for a subunit vaccine would be a neutralizing epitope present in all the isolates, the finding that different neutralization epitopes exist in different isolates could make it necessary to develop vaccines for different geographic areas or to include several neutralizing epitopes in a single vaccine. In the future, it will be important to sequence the genomes of a number of IHNV isolates to deduce which genomic mutations have produced the antigenic differences defined by the MAbs and to determine whether any of the epitopes identified by the MAbs correlate with their virulence for particular species of salmonids.

Further development of MAbs to other epitopes on viral proteins and of methods for rapid sequencing of the isolates will increase our ability to determine the relatedness of viruses associated with fish disease outbreaks. Until that time, only limited conclusions on the epizootiology of outbreaks of these viral diseases in new geographic regions will be possible.

MAbs have also been useful for taxonomic studies of bacteria. MAbs against *Vibrio* sp (Goerlich, 1987; Espelid *et al.*, 1988; Chen *et al.*, 1992) allowed the establishment of *V. salmonicida* as a unique *Vibrio* species, although serotypically related to *V. fischeri* and to *V. anguillarum* (Espelid *et al.*, 1988; Bogwald *et al.*, 1990). MAbs to *V. anguillarum* 0-group 2 were used to compare the cell surface determinants of *V. anguillarum* 0- groups 1 and 2, and *V. ordalii* (Svendsen and Larsen, 1988). A very low cross-reactivity with *V. anguillarum* 01 and V. *ordalii* was observed. This has important implications for vaccination because many vaccines used *V. ordalii* as the representative of serogroup 02. Heterogeneity among strains of *Aeromonas salmonicida*, the etiologic agent of furunculosis, was investigated with 4 MAbs developed against LPS from *A. salmonicida salmonicida.* LPS from isolates of *A. salmonicida salmonicida* (n=10) and *A. salmonicida masoucida* (n=2) reacted with all the MAbs, while 6 isolates of *A. salmonicida achromogenes* failed to react with one of these MAbs (Rockey *et al.*, 1991).

DEVELOPMENT OF FISH VACCINES

Characterization of relevant epitopes for the development of vaccines

Initial identification of potential host-protecting pathogen determinants by the use of MAbs has been made mostly by *in vitro* cell neutralization in the case of virus infectivity. Neutralizing MAbs have been obtained against IHNV (Winton *et al.*, 1988, Ristow and Arnzen, 1991), IPNV

(Caswell-Reno *et al.*, 1986; Christie *et al.*, 1990; Tarrab *et al.*, 1993), CCV (Arkush *et al.*, 1992) , and VHSV (Lorenzen *et al.*, 1990; Sanz and Coll, 1992b).

The glycoprotein G of IHNV and VHSV elicits neutralizing Ab and protective responses (Engelking and Leong, 1989). Localization of its epitopes can be studied using escape mutants resistant to MAb neutralization (MAR mutants). These escape mutants are virus variants that usually do not bind the neutralizing MAb and thus escape neutralization. However, Ristow and Arnzen (1991) found that several of their neutralizing anti-IHNV MAbs, although reactive with IHNV isolates by immunofluorescence assays, were not able to neutralize the isolates, indicating that binding by a MAb does not guarantee neutralization by that MAb. Sequencing of the wild type and of the MAR mutant genomes could map the mutations that have produced the antigenic differences defined by the MAbs. MAR mutants of IHNV were selected by repeated cloning of wild-type virus in the presence of the neutralizing MAbs (Roberti, 1987). When tested in trout, some of the variants showed higher virulence than the original isolate while others showed similar or lower virulence, but no sequences of these mutants have been reported yet.

Identification of immobilization antigens of *Ichthyophthirius multifiliis*, supposed to play a relevant role in protective immunity to this parasite, was undertaken by producing immobilizing MAbs (Lin and Dickerson, 1992). Two antigens of 48 and 60 kDa were identified. Passive transfer of these MAbs to fish was able to confer protection against parasite challenge.

MAbs have been also used to assess the specificity of fish immune responses after natural infection or immunization by assaying the ability of the fish serum samples to compete with the binding by antigen of a MAb with a defined specificity. For instance, Espelid *et al.* (1987, 1988) found that 95% of *Vibrio*-infected salmon Abs bound to the outer surface antigen of *V. salmonicida*..

Vaccine development can be aided in other ways by the use of MAbs. One of the major problems in vaccine control of bacterial diseases is the stability of the bacterial strain used for production. MAbs can be used as very specific reagents for quality control of vaccines because they allow detection of minor changes in surface antigens (Svendsen and Larsen, 1988). MAbs have also been used to purify viral proteins by affinity chromatography of solubilized VSHV (Lorenzen, 1992). This technique could be scaled up for the purification of recombinant viral protein fragments expressed in bacteria, yeast or baculovirus, for use as subunit vaccines or in diagnostic assays.

Passive protection against *in vivo* infection

This method has been applied almost exclusively to viral infections. Although protective epitopes of viruses are often defined by *in vitro* MAb neutralization, this may not always reflect the *in vivo* situation. Passive immunization with MAbs followed by virus challenge appear to be a valuable alternative to *in vitro* neutralization for the identification of the epitopes involved in Ab-mediated mechanisms of control of viral infection.

Examples of this method have been described for VHSV and CCV. Two MAbs (I and II) were able to confer immunity to VHSV infection when administrated intraperitoneally to trout, although only MAb I could neutralize viral infectivity *in vitro* (Lorenzen *et al.*, 1990). These differences may reflect the involvement of different mechanisms for protection *in vivo*. Passive transfer of sera with neutralizing activity was also shown to protect juvenile channel catfish from lethal challenges of CCV (Hedrick and McDowell, 1987).

Selection of best recombinant proteins for vaccine trials

Neutralizing MAbs have been extensively used to select bacterial and yeast clones expressing fragments of VHSV in the right conformation (Estepa, 1992; Estepa and Coll, 1992; Estepa *et al.*, 1994: Lorenzen *et al.*, 1993). Recombinant viral protein-fragments that were recognized by the anti-VHSV MAb panel induced the highest percentage of survival *in vivo* and were stimulatory to the trout leucocyte cultures *in vitro*. In the same way, Gilmore *et al.* (1988) expressed the glycoprotein of IHNV in *E.coli* as a fusion protein. Neutralizing MAbs recognized the fusion protein in blots. Although sera from rabbits immunized with this fusion protein were weakly neutralizing in an *in vitro* plaque-reduction assay, immersion immunization trials of fish with recombinant bacterial lysates induced good protection against IHNV challenge (Xu *et al.*, 1991).

Lawrence *et al.* (1989) expressed the outer capsid protein, VP2, of the infectious pancreatic necrosis virus (IPNV) in *E. coli* as a fusion protein. This fusion protein reacted in blots with a neutralizing anti-VP2 MAb which neutralized the infectivity of 11 out of 12 different IPNV serotypes. Sera from rabbits immunized with the fusion protein reacted with the VP2 polypeptide in blots and with purified virus in ELISA and were able to neutralize IPNV infectity in a plaque-reduction assay, indicating that it could be a potential subunit vaccine against IPNV.

FUTURE PROSPECTS

Although many basic studies remain to be done in lower vertebrate immunology, further development of MAbs for aquaculture will still have new applications in diagnosis and in the development of recombinant vaccines.

The development of MAbs to fish leucocyte/lymphocyte markers is, and probably will continue to be, an area of research that leads to more applications for MAbs in aquaculture. They could be used to monitor fish health by blood analysis and to study cellular immune responses. The application of flow cytometry, commonly used in human pathology and diagnosis, will facilitate the development and selection of more MAbs against new fish leucocyte markers.

In the diagnostic field most of the new developments are expected to burst from the coincidence of a number of technologies such as, MAbs, ELISA, PCR and solid-phase automation. The new refinements in all these technologies could improve specificity and sensitivity to such a high level that fish carrying low concentrations of pathogens, but otherwise

healthy, could be detected and eliminated to prevent dissemination of diseases when stocks were moved. The monitoring of potentially disease-carrier fish eggs to be exported/imported could have an enormous economic impact on aquaculture.

Finally, new ways to obtain MAbs, such as cloning and expression of the variable domains of Igs in bacteria, could probably bypass the difficulties encountered with some antigens (i.e. fish leucocyte markers) and reduce the labour-intensive endeavour of producing hybridomas of the desired specificity.

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