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TELEOST ANTIBODY STRUCTURE: SIMPLE PROTOTYPE OR ELEGANT ALTERNATIVE ?

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Abstract
Teleosts possess mechanism(s) by which they can generate considerable structural diversity within their tetrameric antibody molecules. In salmonids, this diversity is generated through a process of random polymerisation of the constituent monomeric subunits rather than dependency upon isotypic gene diversity. Thus, one gene product can give rise to as many as six different structural forms of immunoglobulin. In contrast to mammals, evidence suggests that this polymerisation process occurs late in the secretory process and not within the endoplasmic reticulum. This assembly process is likely to be important in the generation of teleost antibody functional diversity, thereby potentially simulating isotypy.

Introduction
There are a number of fascinating questions that arise when considering the structure of teleost antibody, both those of a basic nature (e.g. relating to phylogeny, cell biology, etc.) and those of an applied nature (relating to vaccinology and disease resistance). Historically, the opinion has been that the rather restricted isotypic profile of teleost fish somewhat reflects a phylogenetic hierarchy, wherein extant fish species represent more primitive, extinct ancestors. This has been primarily due to their assumed simplicity, while mammals and other vertebrate groups exhibit a level of complexity that is equated with greater evolutionary sophistication. However, since all extant species have been subject to evolutionary processes for the same amount of time, it is far more likely that the need for immunologic complexity to address the selective pressures of disease susceptibility would not leave whole groups of taxa without the necessary functional diversity to survive. Perhaps, we should not accept the paradigm that a diverse collection of CH genes, or variation of molecular weight constitutes the most important measures of the structural or functional potential of a taxon's immune diversity. Therefore, although texts have stated that mammals possess the greatest number of isotypes, while birds, reptiles, and amphibians, an intermediate number, and teleost fish, only one (Horton and Ratcliffe, 1993), perhaps another basis of structural / functional diversity can be examined that is more relevant to the animal under study. Such an approach should not only aid comparative immunologists in more fully assessing the problem and function of isotypy, but may also aid pathologists in dissecting some of the more persistent problems in fish vaccinology, disease resistance, and immunopathology.

Structural Diversity of Teleost Immunoglobulins
In gross structural terms the bulk of all teleost serum Ig is in the form of a tetramer of approximately 800,000 d. Although there have been reports of some serological diversity (Lobb and Olson, 1988; Sanchez *et al.*, 1989; Killie *et al.*, 1991), confirmation of this diversity as being comparable to classical isotypes and not residing within VH regions, or carbohydrate epitopes has not been forthcoming. Further, molecular evidence of specific CH genes encoding these serologically defined isotypes has yet to be demonstrated. However, recent studies have provided some molecular evidence for alternate CH genes, with Atlantic salmon (*Salmo salar*) possibly possessing two forms of δ H chain genes (Hordvik *et al.*, 1992), and channel catfish (*Ictalurus punctatus*) possessing a potential δ H chain gene (Wilson *et al.*, 1997). Largely based on the structure of expressed immunoglobulin, the teleost antibody is composed of four monomeric subunits, each consisting of two identical H chains of approximately 70,000 d and 2 light chains of approximately 25,000 d (Wilson and Warr, 1992). The assembly of these H and L chains into the mature tetrameric antibody, however, can provide considerable heterogeneity for the final product, both within an individual, and also between species. Among all teleosts examined thus far, single heavy and light chains are, initially and uniformly, disulfide-crosslinked into halfmeric forms. However, the remaining employment of disulfide bonds to achieve the final tetrameric product manifests distinctive species variability (Kaattari *et al.*, 1998). The channel catfish appears to display the greatest heterogeneity of oxidised or reduced cysteines (redox forms) within the assembled antibodies (Lobb and Clem, 1983). Serum antibodies from these fish are composed of tetramers which are either fully crosslinked, or composed of various non-covalent combinations of half-, mono-, 1-1/2-, di, 2-1/2-, tri-, and 3-1/2-mers. These latter, non-covalent , assemblages of tetramers possibly possess the potential to exist in any combination of these various forms. Generating structural diversity via a process of differential disulfide bonding is not unique to the catfish, but also occurs within other teleost species, albeit utilising different construction themes. For example, in salmonids (*Oncorhynchus mykiss* and *Oncorhynchus nerka*) the basic covalent subunit is the monomer, with the final tetrameric product being composed of either fully crosslinked tetramers, or non-covalent associations of trimers, dimers, and monomers (Kobayashi *et al.* 1982; Sanchez and Dominguez, 1991; Kaattari *et al.*, 1998; Evans *et al.*, 1998). In the toadfish (*Spheroides glaber*) the tetramer can be either fully crosslinked or employ combinations of dimers and monomers (Warr, 1983), while the sheepshead (*Archosargus probatocephalus*) tetramer can be either fully crosslinked or be composed of dimeric subunits (Lobb and Clem, 1981a; Lobb and Clem, 1981b). It has been observed in trout that the ratio of these forms within a population of immunopurified serum antibodies is fairly constant, regardless of the specificity of the antibody or individual fish from which they were procured (Evans *et al.*, 1998). Therefore, the assembly process, as a whole, appears to be regulated to achieve a consistent ratio of redox forms. In summary, although teleost fish may appear to be primarily relegated to the use of a single, classically defined isotype, this immunoglobulin exhibits a broad range of structural heterogeneity. This structural heterogeneity has tremendous implications not only for potentially diverse effector functions, but also for unique mechanisms of multimeric antibody assembly.

The Uniqueness of the Teleost Antibody Assembly Process
Initially the occurrence of structurally distinctive redox forms of antibody could be considered as evidence that these different tetramers were distinct isotypes. That is, the possibility had to be initially entertained that differential

disulfide crosslinking could reflect the presence of H chains possessing unique, but previously unrecognised differences in the H chain sequences. Two different approaches have been employed to resolve this question.

In the trout, it was reasoned that if the different forms of Ig represented different isotypes, then each form would have to be expressed as a distinct product of a B cell clone. As the isoelectrofocusing (IEF) spectra of immunopurified fish antibodies, including trout, are fairly restricted (Cossarini-Dunier *et al.*, 1986; Wetzel and Charlemagne, 1985), IEF should easily reveal whether each B cell clonal product (isoelectropherotype) possesses only a single form (i.e. isotype), or all forms. Thus, if these different forms are not isotypic in origin but rather are produced by every B cell (i.e. not dependent upon unique H chains), then the isoelectric spectrotype should demonstrate all forms at each pI. This is precisely what was observed (Kaattari *et al.*, 1998). Therefore, not only is there great structural diversity in the tetramer population, but each B cell produces and secretes this same diversity of forms using the same H chain. The secretion of ostensibly incompletely crosslinked IgMs during the assembly process was not anticipated, as an essential role of the endoplasmic reticulum (ER) has been assumed to be exertion of rigorous quality control measures which insure that multimeric immunoglobulins are fully crosslinked or polymerised, or else retained and / or degraded (Hurtley and Helenius, 1989; Fra *et al.*, 1993).

The ability of individual lymphocytes to employ a single H chain gene product in the construction of its immunoglobulin, yet do so with such structural diversity has also been demonstrated in a more elegant, molecular model (Ledford *et al.*, 1993). In this study a chimeric H chain gene was constructed of the CH segments of the catfish H chain gene and a murine VH gene. This construct was then transfected into a murine myeloma cell. Instead of producing a murine-like, fully-polymerised multimer, the mouse cell produced all the structural forms observed in the catfish. This experiment demonstrated two important facts, 1) as in the IEF experiment, each lymphocyte has the capacity to produce all redox forms utilising a single H chain (i.e. the diversity is not due to isotypic differences), and 2) fish lymphocytes are not unique in their ability to produce this redox heterogeneity. Given a catfish H chain, a mouse will assemble IgM in the manner of a catfish. Corroborative of the Ledford study is the work of Guenzi *et al.* (1994) which has provided evidence that the retention of murine monomeric IgM can be affected by insuring that the amino acid residues proximal to the C-terminal cysteine are not acidic (via *in situ* mutagenesis). Interestingly, although most vertebrates possess at least one acidic residue in this region, at least two teleosts, trout (Lee *et al.*, 1993; Andersson and Matsunaga, 1993) and catfish (Ghaffari and Lobb, 1989) do not. This is even more striking when the elasmobranch is considered, which does possess a proximal acidic residue to the C-terminal cysteine and, correspondingly, secretes a fully polymerised, pentameric IgM (Marchalonis and Edelman, 1965; Clem and Small, 1967).

This unique ability to assemble and secrete these varied redox forms of the IgM molecule has prompted our investigations into the possible intracellular mechanisms employed to accomplish this. In the trout we were intrigued by the fact that the ratios of the covalent forms (tetramer, trimer, dimer and monomer) are produced in constant ratios (Evans *et al.*, 1998). Such a consistency in ratios suggested that a well-prescribed process was occurring and that this process might be conducive to mathematical modelling. A number of models were devised to determine if the ratios observed *in vivo* could be simulated and, thereby heuristically provide us with some insight as to the assembly process. Among these models were those that assumed a state of dynamic equilibrium between the redox forms as well as kinetic-based models wherein disulfide bonds would accumulate irreversibly.

Analysing these models revealed that only a kinetic-based model would predict the observed *in vivo* ratio of forms (Evans *et al.*, 1998). However, the biology of such a situation implied that the final disulfide bonding process would likely occur within the secretory vesicle and not the endoplasmic reticulum, the latter occurring in mammals (Hurtley and Helenius, 1989; Fra *et al.*, 1993). We suggest this possibility since if such a process came to completion early in the biosynthetic / secretory pathway (within the ER), then we should expect to see this same ratio of forms within the intracellular contents of activated lymphocytes, however, only monomeric forms are seen (Kaattari *et al.*, 1998). Secondly, only late stage organelles in the secretory pathway receive a bolus of molecules which can be post-translationally modified as a bolus, then secreted. Transport of proteins out of the ER or the Golgi occurs in small quanta of the material, not by the complete liberation of the entire organellar contents as occurs within the secretory vesicle. Thus, in light of our model, the secretory vesicle would be more logical compartment for final assembly.

The above observations and rationales have other, deeper implications. For example, does the placement of select amino acids near a particular cysteine influence not only the degree of disulfide bonding, but also the means and mechanism underlying intracellular transport and secretion? Another question that arises is what residues primarily determine the structural architecture of multimeric Igs in these animals? If this process occurs within the secretory vesicle, then are the relative ratio of forms dependent upon the regulation of protein disulfide isomerase (PDI) or similar enzymes? Are enzymatic inhibitors or activators induced during an immune response which modify these ratios? Being an enzymatic reaction, could changes in the ambient temperature affect the relative ratio of forms within these ectothermic species? Indeed, there appear to be a number of venues by which structural diversity of teleost Igs could be altered and if these forms are responsible for different functional activities, then parameters such as temperature and PDI induction may dictate the teleost equivalent of isotype switching.

Functional Implications of Redox Structural Diversity

Binding site functionality

The physical arrangement of antigen-binding sites (Fabs) on the various mammalian isotypes allow these species to bind a range of epitope spatial arrays. For example, although the mammalian IgM Fc is fairly rigid (Nezlin, 1990), possession of ten equally spaced binding sites, provides the molecule with considerable spatial latitude in binding multivalent antigens. Thus possession of a greater span, together with multiple binding sites, provides the IgM molecule with the ability to bind with the extremely high avidity (Crothers and Metzger, 1972). Conversely, although IgG and some IgA antibodies may be flexible, they do not possess a span or valency which would permit as much crosslinking as seen with IgM. However, given the process of somatic mutation and antigen-driven selection,

the individual binding sites of IgG or IgA may be able to achieve equivalent intrinsic affinities that are high enough to bind epitopes with an avidity comparable to that of IgM. In the case of teleost fish, a single set of structurally diverse tetramers may easily provide a combination of binding capabilities not available to mammals. For example, the capacity to crosslink two or more epitopes is not likely to be solely determined by the reach of a single, rigid, fully crosslinked tetramer, but rather by a suite of antibodies with varying degrees of flexibility. Thus, the teleost may achieve the specific span needed to accommodate any array of epitopes. As each set of tetramers produced by a single B cell should, therefore, exhibit varying capabilities for crosslinking, it may be that a tetramer composed of non-covalently associated monomers would have the flexibility to 'reach' epitopes too far outside or too closely within the reach of a more rigid form. In this context, it might stand to reason that antibodies reactive with, for example, carbohydrate residues on bacterial polysaccharides may preferentially engage only a select form(s) of antibody, whereas a comparable epitope expressed on a glycoprotein would be preferentially bound by another form. The flexibility in accommodating differentially spaced epitopes could also serve to increase the avidity of binding over a more rigid, fully-polymerised form. A more flexible array of Fabs would be able to accommodate more epitopes by physically adjusting in orientation to accommodate them. This capability would enable such an antibody to bind more epitopes and thereby increase the multi-point attachment of the antibody. This should, in theory, permit more avid binding for the more flexible forms of antibody.

Alternatively, since it is likely that specific kinds of epitopes are likely to be primarily expressed in different spatial formats (i.e. polysaccharide epitopes on bacterial surfaces, or protein epitopes on viruses or toxins), it could be that the antigens themselves, depending whether they are protein vs. polysaccharide, or cellular vs. soluble, could induce auxiliary signals that modulate PDI production or activity and thereby regulate the degree of crosslinking within a population of antibodies. Unfortunately the bulk of the physico-chemical analysis on teleost antibody redox structure has focused only on the antibodies induced to soluble protein antigens. Comparative studies should be pursued to examine possible differences in response to, for example, haptens conjugated to different carrier forms, such as bacteria, viruses, proteins, polysaccharide, and synthetic carriers.

Fc Functionality

The Fc portion of the antibody molecule is essential in mediating a variety of effector functions. For example, the Fc region of mammalian IgM is particularly effective in activating complement, primarily due to its multivalent nature. Although mammalian IgM is a primarily pentamer and C1q (the first component of the complement cascade to interact with antigen-bound IgM) is hexameric, it has been found that a certain portion of this IgM is produced as a hexamer and that this form is over 100-fold more effective in activating complement (Wiersma *et al.*, 1998). Thus, the fact that most IgM is pentameric implies that the production of the form of mammalian IgM (pentamer vs. hexamer) may be regulated to modulate the degree of complement activation. By the same token the flexibility of the monomeric tailpieces within of teleost IgM Fc may regulate the activation of teleost complement. The spacing of the epitopes upon a rigid structure could be transduced by the flexibility of the bound IgM into a range of affinities for teleost C1q and its subsequent activation as has been postulated by studies with murine IgM. Through the use of *in situ* mutagenesis, conversion of Cys 414 to Ser 414 has been found to completely eliminate complement activating function of murine IgM (Davis *et al.*, 1989; Arya *et al.*, 1994). Feinstein *et al.* (1986) postulated that a loss in IgM rigidity during binding to multivalent epitopes could result in conformational changes that could prevent C1q binding and subsequent activation of the complement pathway. Interestingly, this sensitivity to free sulfhydryls has even been observed in IgG mediated complement fixation (Brekke *et al.*, 1995), thus the simple redox state, as opposed to more global conformational changes may also regulate these functions as well.

Alternatively, although a particular spatial orientation of the antigen-bound Fc may be ineffective in activating complement, it may be effectively suited to arming macrophages or other phagocytic, granulocytic, or lymphocytic cells. Thus far, the only reports of Fc-mediated cellular functions in fish have been for opsonization (Griffin, 1983; Whyte *et al.*, 1990; Rombout *et al.*, 1991; Lamas and Ellis, 1994); however, other comparable functions may also be mediated through interaction with Fc receptors (FcRs) on various cell types. Thus it may be possible that specific effector functions may be elicited simply through the preferential interaction with a specific redox form, or, alternatively, through an antigen-induced rearrangement of a flexible Fc.

Importance to Fish Health Research and Clinical Practice

To date measurement of antibody responses to fish pathogens has relied simply upon the estimation of titer by agglutination, precipitation, or by ELISA techniques. Considering the possibility that the redox form of the antibody may determine the effectiveness of the response, knowledge of the antibody titer may bear little relevance to the state of immunoprophylaxis. For example, if effective immunity to a particular bacterial pathogen requires a particular flexible redox form which cannot be serologically distinguished from all other forms, a high antibody titer may not correspond with immunity if that specific redox form is not present. Therefore, in such a situation, one might wrongly assume that antibodies are not important, or even antagonistic to protective immunity. Alternatively, if a vaccine is prepared that induces only low amounts of a needed redox form, one may come to the conclusion that vaccination, in general, is not prophylactic. Conversely it may be possible that under certain physiological conditions that could alter the distribution of redox forms, that the same vaccine may generate large quantities of the needed redox form, and, thus possess greater efficacy. Such physiological modulation might be achieved by simply altering the temperature subsequent to vaccination, or by employing chemotherapeutics that lead to up-regulation of PDI. Thus, for a specific antigen, it may be necessary to ascertain the relative ratios of the antibody forms induced and determine which forms manifest the greatest level of protection for a specific pathogen. With this knowledge, we may be able to explain and circumvent the numerous situations reported in the literature where high antibody titers appear to bear no relationship to prophylaxis (Evelyn, 1971; Olivier *et al.*, 1985; Cipriano and Ruppenthal, 1987;

Bruno, 1987) and , in some cases, are indicative of poor prognosis (Piganelli *et al.*, 1999).

It also stands to reason that the simple induction of a protective redox form may not be sufficient to insure protection. If all forms are produced, while only one is protective, the other forms could effectively block the effective employment of the efficacious form. This concept of blocking antibodies has been described previously in mammals with respect to interference with effective anti-cancer immunity (Brown *et al.*, 1978) and the suppression of atopic reactions (Kuby, 1992). Although these specific situations refer to the blocking of an effectual antibody of one mammalian isotype with that of another, or antibody blocking of cellular immunity, if certain redox forms are incapable of mediating a specific function, they may block the form(s) which can perform that function. In this case an inappropriate vaccine may induce too high a concentration of ineffectual, or blocking antibodies. Alternatively, another reason that a normal immune response to a pathogen may prove ineffective could be that the antigen array on the pathogen, or the manner in which the pathogen biologically modulates the B cell response may result in an elevated amount of this blocking antibody form.

Future Directions

Of paramount importance in understanding the structural / functional correlates of the teleost antibody will be an in depth exploration into the role these different forms of immunoglobulin may play in immunity. Additionally, if these forms are functionally different, the next logical focus would be to determine if the ratios of forms can be skewed by the forms of immunogen, environmental effects (i.e. temperature), or via the use of chemotherapeutics. Concomitant with such analysis would be the development of the means to readily and efficiently titer these individual forms. Although, at present, one can distinguish the relative ratios of redox forms of serum antibody, this analysis requires that the antibody be immunopurified, then subjected to denaturing, non-reducing, composite gel electrophoresis followed by densitometric analysis (Evans *et al.*, 1998). A rapid analysis (as with ELISA) of the concentration of each form would be the most practical and effective means of assessing the titers of these forms and might become comparable to the measurement isotype-specific titers in man and animals. Unfortunately, all the major antigenic epitopes on each of these redox forms would be shared as they are the product of the same H chain. In order to serologically distinguish these forms, the only alternative would be to distinguish epitopes that are distinctively related to the presence of specific disulfide bonds. This would be a fairly difficult task, however such monoclonals have been developed to other protein antigens (Wan *et al.*, 1995). Secondly, if these epitopes are not highly immunogenic, the method of subtractive immunisation (Matthew and Sandrock, 1987; Williams *et al.*, 1992) might be employed to selectively produce antibodies to these, possibly minor antigenic epitopes. By this method mice could be tolerised to unlinked monomeric forms retrieved from denaturing gels or gel filtration columns. Then, in a subsequent challenge with the fully or partially crosslinked forms, only inter-subunit disulfide-associated epitopes could elicit a response, perhaps yielding antibodies to specific redox forms.

Possession of such diagnostic tools would permit pathologists to re-examine situations wherein antibody titers did not appear to be conducive to immunity, and, more specifically, determine the role of specific redox forms may play in immunity and pathology. Future isolation of redox forms, or their selective elicitation and employment in passive immunisation studies should enable immunologists to determine which forms are protective and which exert antagonistic blocking functions.

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