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Humoral immune response of the small-spotted catshark, *Scyliorhinus canicula*

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ABSTRACT

Cartilaginous fishes are the oldest group in which an adaptive immune system based on immunoglobulin-superfamily members is found. This manuscript compares humoral immune function in small-spotted catshark (*Scyliorhinus canicula*) with that described for spiny dogfish (*Squalus acanthias*), another member of the Squalomorphi superorder, and nurse shark, the model for humoral immunity in elasmobranchs and a member of the Galeomorphi superorder. Although small-spotted catshark and nurse shark are separated by over 200 million years we found that immunoglobulin isoforms are well conserved between the two species. However, the plasma protein profile of small-spotted catshark was most similar to that of spiny dogfish, with low levels of pentameric IgM, and IgNAR present as a multimer in plasma rather than a monomer. We show that an antigen-specific monomeric IgM response, with a profile similar to that described previously for nurse sharks, can be raised in small-spotted catshark. Lacking polyclonal or monoclonal antibody reagents for detecting catshark IgNAR we investigated phage-display and recombinant Fc-fusion protein expression as alternative methods to look for an antigen-specific response for this isotype. However, we could find no evidence of an antigen-specific IgNAR in the animals tested using either of these techniques. Thus, unlike nurse sharks where antigen-specific monomeric IgM and IgNAR appear together, it seems there may be a temporal or complete 'uncoupling' of these isotypes during a humoral response in the small-spotted catshark.

1. Introduction

The cartilaginous fishes are the most ancient lineage in which an adaptive immune system built upon immunoglobulin superfamily molecules (that is to say antibodies, T-cell receptors and major histocompatibility complex), can be found [1]. This group, which includes sharks, skates and rays, diverged from a common ancestor with other jawed vertebrates between 450 and 500 million years ago (MYA) [2,3]. The Holocephali (chimaera and ratfish) and Elasmobranchii (sharks, skates and rays) lineages emerged ~420 MYA, with the latter lineage eventually giving rise to the Galeomorphi and Squalomorphi superorders ~200 MYA [3]. Nurse sharks (*Ginglymostoma cirratum*), members of the Galeomorphi, are, to date, the best studied species of cartilaginous fishes as regards humoral immunity and thus serve as the model for this group. However small-spotted catsharks (*Scyliorhinus canicula*), the subject of this work, are members of the Squalomorphi, thus the evolutionary distance between these species is roughly equivalent to that between the egg-laying prototherian mammals (monotremes such as the platypus) and the live-bearing therian mammals (marsupial and placental mammals).

To date, three immunoglobulin (Ig) heavy chain isotypes have been identified in cartilaginous fishes; IgM, IgW and IgNAR [4]. Shark IgM is orthologous to that of mammals however in all species of shark examined so far, it is found in the blood in two forms, as both a monomer (mIgM) and a pentamer (pIgM). As in mammals, shark pIgM is composed of five IgM molecules (thus having 10 binding sites) linked by the J-chain. In nurse shark pIgM appears almost innate in character; it binds with low affinity but high avidity, does not undergo affinity maturation and does not appear to require T-cell help. In contrast mIgM is thought to be the functional equivalent of mammalian IgG, giving a highly antigen-specific T-dependent response, with evidence of immunological

Abbreviations: aa, amino acid; C, Ig constant region; H, heavy chain; Ig, immunoglobulin; IgSF, immunoglobulin superfamily; h, hour; L, light chain; m, minute; mAb, monoclonal antibody; MYA, million years ago; Sec, secretory tail; Tm, transmembrane region; V, Ig variable region.

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memory [5,6]. The two forms of IgM are produced independently (i.e. neither is formed through the polymerisation or dissociation of the other [7]) and, it is thought, by different shark B-cell lineages [5]. Shark IgW is the orthologue of mammalian IgD [8] and is found in two forms. The long form, with six constant domains possesses a canonical secretory tail like shark IgM and IgNAR. In contrast the short form has only two constant domains and an unusually long secretory tail with multiple cysteine residues and a potential N-linked glycosylation site [9]. Unusual IgW transcripts, in which the variable (V) region has been removed from the long form by alternate splicing (IgWΔV), have also been found in two shark species [10]. The function of IgW in cartilaginous fishes is, as yet, unknown; however, due to their very different molecular structures, it is likely that the long and short forms have distinct roles. The final isotype, IgNAR, is unique to cartilaginous fishes and consists of a heavy-chain homodimer that does not associate with light chains [11]. The binding (V) domains, which are attached to the constant domains via a highly flexible hinge, are highly soluble and do not require dimerisation for high-affinity antigen binding [12,13]. In nurse shark, the IgNAR response is T-dependent and highly antigen-specific [5]. A role for IgNAR that is distinct from that of mIgM has yet to be proven, however differences in structure and tissue distribution [9] strongly suggests that the two isotypes have different effector functions, as is found for different isotypes in mammals.

A recent study examining the Ig repertoire of the spiny dogfish (*Squalus acanthias*), also a member of the Squalomorphi, reported that all the isoforms described in nurse shark were also found in this species. However there were some notable differences in their expression in blood, in particular, IgNAR was found to be present in a hitherto undescribed multimeric form [10]. Unfortunately this study did not address the immune response or functional role of the Igs in spiny dogfish.

In this manuscript, we set out to characterise the Igs of small-spotted catshark and begin to assess the humoral immune response of this species. In the absence of a monoclonal antibody against small-spotted catshark IgNAR we also investigated phage-display and recombinant Fc-fusion protein expression as alternative methods to look for antigen-specific responses.

2. Materials and methods

2.1. Animals

Wild small-spotted catsharks (*S. canicula*) were obtained from sea lochs on the west coast of Scotland and maintained at the Marine Laboratory, Marine Scotland, Aberdeen, UK. Adult animals were held in large, indoor tanks supplied by flow-through seawater ranging in temperature from 5 to 14 °C. Animals were anesthetized with MS-222 (0.12–0.16 g/L seawater) prior to any procedure; bleeds were harvested from the caudal vein into ~1/10 blood volume of porcine heparin sodium salt, reconstituted in elasmobranch-modified PBS (see Section 2.8) to 1000 U/ml, then spun at ~300 g for 10 m to isolate blood plasma. All procedures were conducted in accordance with the UK Home Office 'Animals and Scientific Procedures Act 1986' and Pfizer Corporate Policy #507 on animal care and use.

2.2. SDS-PAGE analysis of small-spotted catshark plasma proteins

Blood plasma from small-spotted catshark, spiny dogfish and nurse shark were each mixed with a sufficient volume of non-reducing 2× Laemmli sample buffer (4% SDS (w/v), 20% glycerol (v/v), 0.004% bromophenol blue (w/v) dissolved in 0.125 M tris (pH 6.8), so that 1 μl of plasma could be loaded per well for each sample. Spiny dogfish plasma was obtained as part of a previous study [detailed in [10]] and nurse shark plasma kindly gifted to us by Martin Flajnik (University of Maryland at Baltimore, USA). Samples were boiled prior to loading onto a 6% tris-glycine SDS-PAGE gels and running in TGS buffer for 1.5–2 h at 100 V. Protein bands were visualised via Coomassie blue staining.

2.3. Total RNA and mRNA production

Total RNA was prepared from the major lymphoid organs freshly harvested from adult catsharks following mechanical disruption in TRIzol reagent according to previously published methods [10]. The RNA pellets were resuspended in an appropriate volume of RNase-free H_2O, the OD260/280 taken to quantify the sample and the RNA quality checked on a denaturing agarose gel before storage at −80 °C for future use. Where required, mRNA was prepared from total RNA using MicroPoly(A) Purist kit (Ambion) according to the manufacturer's protocol.

2.4. Genomic DNA production

Catshark gDNA was prepared from 100 μl (~3×10^6 cells) heparinised, packed red blood cells using previously published methods [10].

2.5. cDNA synthesis, PCR and RACE, cloning for sequencing

Oligo-dT primed cDNA was synthesised from 1 μg total or mRNA using Illustra Ready-to-Go RT-PCR beads (GE) according to the manufacturer's protocol and primed using the oligo-dT primer supplied with the kit. Rapid Amplification of cDNA Ends (RACE) 5′ and 3′ reactions were conducted using SMARTer RACE cDNA amplification kit (Clontech) according to the manufacturer's protocol. Generally a nested approach was required, utilising gene specific primers with the supplied 'universal primer A' for the first round and the 'nested universal primer' (NUP) for the second round. To enable the cloning of small-spotted catshark IgNAR we aligned the sequences from all species available and designed degenerate primers against highly conserved regions in framework region (Fr)1 of the variable region, and in constant (C) domains 1, 3 and 5. Some primer pairings yielded partial IgNAR sequences from which gene-specific primers were designed to complete the sequence; all gene specific primers are detailed in Table 1. PCR products were run on 1% agarose gels and the desired bands cut out, gel extracted and cloned into the pGEM T-easy vector (Promega) to enable sequencing with the vector-specific primers T7 and SP6. A similar strategy was also used to sequence IgM and IgW from this species.

2.6. Analysis of IgNAR transcripts

To look at the levels of full length IgNAR and the new IgNAR transcript which lacks a variable region (IgNARΔV) we used the primers scNAR-leaderF1 with scNAR-secR1 on oligo-dT primed whole blood and spleen cDNA. The PCR product was cloned into pGEM T-easy vector and resultant clones screened with primers against the leader (scNAR-leaderF1) and C2 domain (scNAR-C2R1). Products of two sizes were observed and sequencing of individual clones confirmed these corresponded to the full length and ΔV form of catshark IgNAR.

2.7. Southern blot

Each of the restriction enzymes *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Sac*I, *Spe*I and *Pst*I were used to digest 10 μg of catshark gDNA, the