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Original article

Determination of immunoglobulin novel antigen receptor (IgNAR) *in vivo* affinity maturation in brownbanded bamboo shark (*Chiloscyllium punctatum*)

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Introduction

Immunoglobulin novel antigen receptor (IgNAR) composed of one variable and five constant domains which exists in serum of sharks at low concentrations of 0.1 – 1.0 mg/ml [1]. Affinity maturation studies play a major role to understand the functions of IgNAR and to determine its potential as an immunotherapeutic. The first IgNAR clone with an affinity against hen egg lysozyme (HEL) was developed by Flajnik and colleagues as a monomeric domain with the affinity of 20 nM by phage display technique [2]. Further studies on hyperimmunization of nurse shark (*Ginglymostoma cirratum*) proved the *in vivo* affinity maturation of vNAR domains showing higher specificity and memory as a member of acquired immune system [3,4].

IgNAR was found in several cartilaginous fish species but only a few of them were tested upon affinity maturation. Although studies on nurse shark, horn shark (*Heterodontus francisci*) and spiny dogfish (*Squalus acanthius*) obtained the affinity maturation in IgNAR successfully, small-spotted catshark (*Scyliorhinus canicula*) did not show response to antigen [5]. Therefore, further investigations on affinity maturation of IgNAR in other shark species need to be performed to enhance knowledge on this field. This study investigated the possibility of raising IgNAR antibodies in brownbanded bamboo shark (*Chiloscyllium punctatum*) against hen egg lysozyme (HEL) through *in vivo* affinity maturation. Smaller size of variable region, high thermal and pH stability and higher affinity [6] makes it a potential candidate as a future immunotherapeutic.

Materials and methods

Five mature sharks were exposed to ~ 250 µg of HEL antigen (Roche, Germany) which injected into the second dorsal fin via subcutaneous route. Blood samples were collected from caudal vein and the antigen was given in combination with Complete Freund's adjuvant (CFA) (Sigma-Aldrich, USA) at first exposure, followed by monthly immunizations of HEL mixed with Incomplete Freunds' adjuvant (IFA) (Table 1).

Table 1. Duration of immunization and sample collection

Month	Sample collected	Immunization
0	Pre-bleeding	Antigen in CFA [1]
1	Post injection 1	Antigen in IFA [2]
2	Post injection 2	Antigen in IFA
3	Post injection 3	Antigen in IFA
4	Post injection 4	Antigen in IFA

[1] Complete Freunds' Adjuvant, [2] Incomplete Freunds' Adjuvant.

Protein analysis was performed using normalized plasma concentrations of 1600 µg/ml. Reducing 10% SDS PAGE gels were used and western blotting was performed using 2% casein as blocking solution. Rabbit polyclonal anti-horn shark IgNar (GeneTex, USA) and goat anti-rabbit IgG (Sigma-Aldrich, USA) were used as primary and secondary antibody respectively. Final reads were obtained by western blot analyzer (Odyssey, Li-COR Biosciences, USA).

Modified indirect enzyme linked immunosorbent assay (ELISA) was developed to investigate the affinity maturation of brownbanded bamboo shark IgNAR antibodies against HEL. ELISA plate composition after adding all reagents was illustrated in Figure 1. The absorbance values were measured at 450 nm using iMark

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microplate reader (Bio-Rad, USA) just before and after adding H_2SO_4.

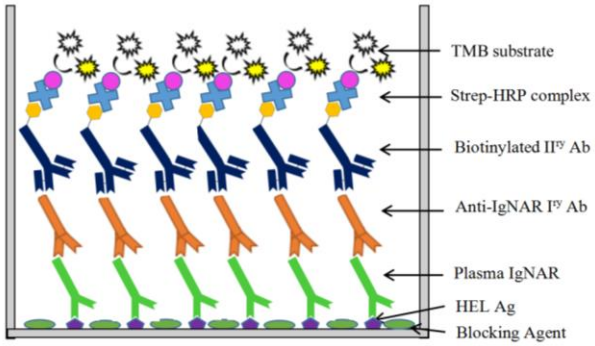

Fig. 1. Schematic illustration of the ELISA designed for the detection of brownbanded bamboo shark anti-HEL IgNAR antibody.

Results and discussion

Western blot analysis revealed bands near 80, 60 and 25 kDa regions. This band pattern might be due to degradation of IgNAR caused by long storage conditions (4 – 8 months at -20°C) [4] which resembles the weight of IgNAR fragments separated at different loops (domain binding sites) as indicated in Fig. 2.

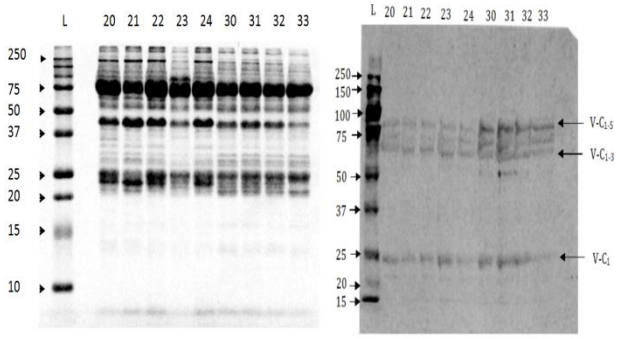

Fig. 2. Plasma protein and IgNAR antibody analysis by SDS-Page (left) and Western blotting (right) results. Sample IDs were indicated on top; 20, 30: pre-exposure, 21-24 and 31-33 samples were exposed to HEL 1-4 and 1-3 times respectively. L-ladder.

ELISA results indicated an increase of IgNAR antibody titer after third exposure to HEL antigen (four months post-exposure) compared to the pre-exposure samples as shown in figure 3. In nurse shark injected with HEL via intra-venous route, IgNAR antibody titer was increased at third or fourth immunization and reached plateau at seven months [4]. But in present study antigen was injected via subcutaneous route at each immunization for slow exposure for prolong period.

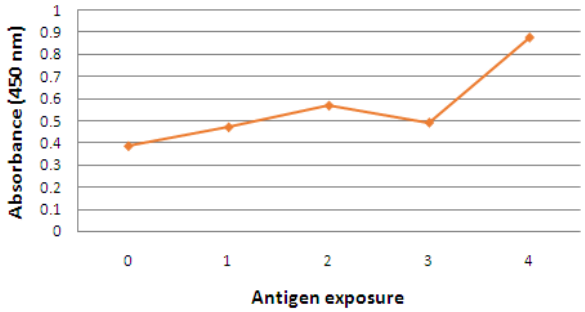

Fig. 3. ELISA absorbance values of anti-HEL IgNAR antibodies in pre and post-exposure samples from brownbanded bamboo shark.

Conclusions

Plasma protein analysis of antigen exposed samples revealed the evidence of *in vivo* affinity maturation in brownbanded bamboo sharks. Hence, they can be used as a model organism to perform affinity maturation studies.

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