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SHORT COMMUNICATION

The spontaneous haemolytic activity of cod serum: Heat insensitivity and other characteristics

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The complement system is an important element of both the innate and adaptive immune system, and the alternative, lectin and classical pathways have been described in fish (Sakai, 1992; Matsushita *et al*., 1998; Sunyer & Lambris, 1998). The alternative and lectin pathways are ancient mechanisms present in invertebrates as well as vertebrates. The alternative pathway is activated non-specifically by various micro-organisms. The lectin pathway, which has not been fully studied in teleosts (Matsushita *et al*., 1998), is activated by the binding of mannose on the surface of micro-organisms to a complex of mannose-binding lectin (MBL) and MBL-associated serine protease (MASP). All three pathways converge to the lytic pathway, leading to opsonisation or direct killing of the micro-organism. The complement system is composed of numerous proteins and all pathways generate factor C3, which has been described and isolated from teleost species (Nakao & Yano, 1998).

Fish complement is comparable to the mammalian system, but some important differences have been described. In general, the complement system of the alternative pathway is more active in fish than in mammals, is more heat labile and therefore difficult to preserve. Also, fish complement has a lower optimum reaction temperature than its mammalian counterpart, and its components, i.e. C3, are more polymorphic (Nonaka *et al*., 1981*a*,*b*; Sakai, 1983, 1984*a*,*b*, 1992; Nakao & Yano, 1998; Sunyer *et al*., 1997; Sunyer & Lambris, 1998).

In recent studies of cod humoral immune components it was noticed that the spontaneous haemolytic (SH) activity of cod, which is generally assumed to reflect alternative pathway activity (Sakai, 1992), appeared to be unusually heat-insensitive and unaffected by storage at −20° C (Magnadóttir *et al*., 1999*a*,*b*). The aim of the present work was to examine this heat insensitivity and other characteristics of the SH activity of cod serum.

The assay and test material

The standard assay of the SH activity was based on a method described by Sakai (1992) with modifications, some of which were adapted for the optimisation of the haemolytic activity of cod serum. The method has been described in detail elsewhere (Magnadóttir *et al*., in press). Preliminary analysis showed that: (i) the optimal assay temperature was 37° C (tested at 4, 15, 22 and 37° C); (ii) ethylene glycol-bis-tetraacetic

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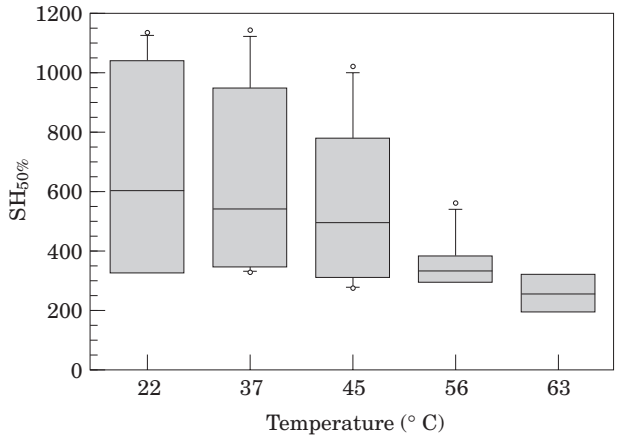

Fig. 1. The effect of temperature on the SH activity of cod serum. Box plots of $SH_{50\%}$ values of six sera incubated for 30 min at different temperatures.

acid (EGTA, up to 10 mM) which binds Ca^{2+} and should inhibit the classical pathway, had no effect on the SH activity; and (iii) ethylene dinitrilo tetraacetic acid (EDTA), which binds both Ca^{2+} and Mg^{2+} and should inhibit both the classical and alternative pathway, enhanced the SH activity. Testing nine sera the mean $SH_{50\%}$ was 202 without EDTA and 469 in the presence of 1 mM EDTA.

Unless otherwise stated the test buffer was prepared from complement fixation test tablets from Oxoid (U.K.) with additional 0·1% gelatin and 1 mM EDTA, and the target cells were red blood cells from sheep (sRBC). Other target cells than sRBC were tested and it was found that RBC from rabbit and rainbow trout were more sensitive to the haemolytic activity of cod serum than sRBC and both types were similarly more sensitive in the presence of 1 mM EDTA. sRBC were used in this study for the practical reason of being easily attainable. The results were expressed as $SH_{50\%}$ values, the serum dilution that gave 50% lysis.

Serum samples from three groups of cod were used: (i) sera with relatively high SH activity ($SH_{50\%} \geq 600$), from wild cod of different sizes (20–100 cm in length) collected in the autumn of 1996 (Magnadóttir *et al.*, 1999*b*) and stored at −20° C; (ii) sera with relatively high SH activity ($SH_{50\%} \geq 800$), from cultivated cod, 1–2 year old, which had been immunised with PBS (0·01 M, pH 7·4 phosphate buffered saline Sigma, U.S.A.), LPH (*Limulus polyphemus* hemocyanin, Sigma) or TNP-LPH (trinitrophenyl haptenated LPH) in FCA (Freund complete adjuvant) and kept at 4 or 9° C for up to 18 months, collected 1996–1997 (Magnadóttir *et al.*, in press) and stored at −20° C; and (iii) sera, with no SH activity ($SH_{50\%} < 4$) from cultured cod which had been kept at 4 or 9° C for about 10 months from hatching, collected in January 1999 and stored at −80° C.

The effects of temperature on the SH activity

The heat sensitivity of the SH activity was tested in six sera from wild cod. These were diluted 10^{-1} in saline and incubated for 30 min at 22, 37, 45, 56 and 63° C and the SH activity then measured (Fig. 1). There was a relatively small reduction in SH activity after incubation at 37° C (about 5% reduction) and at 45° C (about 16% reduction). After incubation at 56° C the SH activity was about 55% of the control and even after incubation at 63° C the mean $SH_{50\%}$ value was 125. Generally, incubation for 20–30 min at 45° C completely abolishes the haemolytic activity of, for example, rainbow trout, goldfish, catfish and tilapia serum (Sakai, 1992).

One of the sera tested above was diluted 10^{-1} in saline, incubated at 56° C, a sample collected at 0, 10, 20, 30, 60 and 120 min, stored on ice, and the SH activity measured at

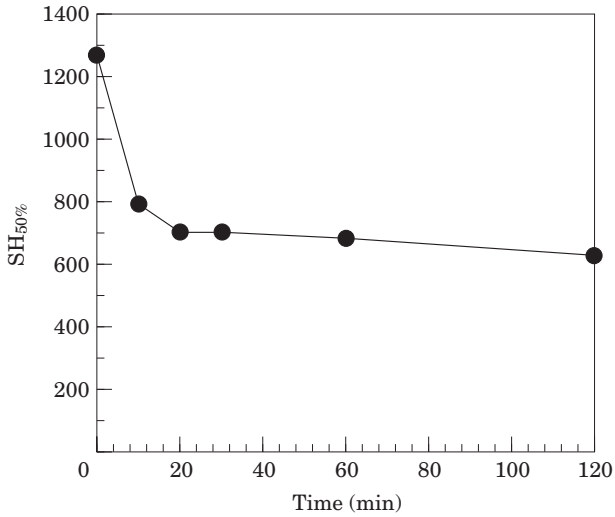

Fig. 2. The SH activity of cod serum incubated for up to 120 min at 56° C before determining the SH activity.

Table 1. The effects of various agents on the spontaneous haemolytic (SH) activity of cod serum

Agent	SH50% before treatment	SH50% after treatment
Zymosan,[a] 10 mg ml^{-1}	1245	500
Asa[b] whole bacteria, 10^8 bacteria ml^{-1}	1110	600
Asa[b] bacterin,[c] 0·4 mg ml^{-1}	1110	640
Asa[b] ECP[d] 0·8 mg ml^{-1}	1110	1180
Asa[b] LPS[e] 3·2 mg ml^{-1}	1825	880
Mouse anti-cod IgM antibody (ascites)[f] 10^{-1}	610	<40[g]
Control ascites[f] 10^{-1}	610	<40[g]
Goat serum[f] 10^{-1}	610	<40[g]
Sea bass serum[f] 10^{-1}	610	<40[g]
SH negative cod serum 10^{-1}	1825	<40[g]
Goat anti-human C3 antibody (Ig) 10^{-1}	2280	1790
PMSF[h] 1 mM	1825	1825

The table shows the $SH_{50\%}$ value before and after incubation with the given concentration of each agent (the results are shown for five individual sera $SH_{50\%}$ 610–2280).

[a]Polysaccharide from yeast, [b]*Aeromonas salmonicida* ssp. *achromogenes*, [c]formalin killed and sonicated bacteria (Asa), [d]extracellular product from Asa, [e]lipopolysaccharide from Asa, [f]inactivated at 56° C for 30 min, [g]detection limit of the assay, [h]Phenylmethylsulfonyl fluoride protease inhibitor.

the end of the 2 h incubation period (Fig. 2). The results show that there was a rapid fall in activity in the first 30 min to a level of about 55% of the original activity and this remained relatively unchanged for up to 2 h.

The effect of different agents on the SH activity

The effects of various agents on the SH activity of cod serum was tested (Table 1). None of these agents had any effect on the sRBC on their own. Some of the agents, like

zymosan and LPS (lipopolysaccharide), are known activators of the alternative pathway of mammalian and fish complement (Sakai, 1983).

Cod sera were diluted in the test buffer, mixed with an equal volume of the appropriate agent, diluted in the test buffer and incubated for 2–3 h at 22° C. Each agent was tested in serial dilutions. Table 1 shows the optimal effect each agent had on the SH activity. Zymosan and LPS reduced the SH activity of cod serum significantly, although not completely. The reducing effect of the whole bacterial preparations of Asa (*Aeromonas salmonicida* ssp. *achromogenes*) may also be attributed to the LPS content of the cell wall. ECP (extracellular products) had little or no effect. Other workers have shown that normal serum from other species interferes with fish complement, but the reason for this is not known (Sakai, 1992). As can be seen in Table 1 the haemolytic activity of cod serum is completely abolished by sea bass and goat serum and also by anti-cod IgM ascites and normal ascites. However, what was surprising was the fact that serum from cod with no detectable SH activity also abolished the SH activity of the test serum.

In view of the importance of serine proteases in the complement pathways the effect of the serine protease inhibitor PMSF (phenyl methyl sulphonyl fluoride) was examined but was found to have no effect (Table 1). However, if EDTA was omitted from the test buffer, PMSF had, like EDTA, an enhancing effect on the SH activity.

Other notable features

Previous studies showed that SH activity was relatively unaffected by the environmental temperature, whereas seasonal factors seemed influential (Magnadóttir *et al.*, 1999*a*,*b*; Magnadóttir *et al.*, in press). It was also observed, when cod was experimentally infected with Asa, that the higher the dose used, the lower the SH activity of the survivors (unpublished data) indicating that infection may influence the SH activity. Preliminary studies have also shown that cod serum does not have a bactericidal effect on Asa *in vitro* (unpublished data). Goat anti-human C3 antibody (Sigma), tested on Western blotting of cod serum with strong SH activity ($SH_{50\%}$=2280) reacted with 75, 47 and 29 kDa bands, whereas only the 75 kDa band was detected in Western blotting of cod serum with no SH activity. The SH activity of cod was generally high ($SH_{50\%}$>200) but very variable both within similar groups of cod and in individuals sampled at different times (Magnadóttir *et al.*, in press). As well as reflecting the sensitivity of this parameter to inherent or external factors this may also be an indication of a polymorphism commonly associated with fish complement factors (Wiegertjes *et al.*, 1993). A complete absence of SH activity was associated with one group of cod which had been hatched at the experimental station and kept at 4 or 9° C for about 10 months. The reason for this is not known but may be linked to a latent infection.

Conclusions

These results demonstrated some features in common with fish complement system, such as the inhibition by zymosan and LPS (albeit limited), the inhibition by serum from other species and the variable RBC type compatibility. Unusual features were the heat stability and the optimal activity at 37° C, and the enhancement of the SH activity by EDTA (and PMSF). These observations suggest that other haemolytic factors may be involved either in conjunction with or independent of the complement system of cod.

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