NOTE

Inhibition of hemolytic activity of *Aeromonas salmonicida* GCAT in rainbow trout red blood cells by a monoclonal antibody

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ABSTRACT: The present study shows that a monoclonal antibody (MAb) directed to *Aeromonas salmonicida* glycerophospholipid:cholesterol acyltransferase (GCAT) is capable of protecting rainbow trout red blood cells from the cytotoxic effects of the enzyme *in vitro*.

KEY WORDS: *Aeromonas salmonicida* · GCAT · Monoclonal antibody

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Glycerophospholipid:cholesterol acyltransferase (GCAT) secreted by *Aeromonas salmonicida* ssp. *salmonicida* in a complex with lipopolysaccharide (LPS) has been characterized as a major lethal toxin for salmonids (Titball & Munn 1981, Buckley 1982, Lee & Ellis 1989, 1990, Arnesen et al. 1993, Røsjø et al. 1993, Eggset et al. 1994). It was suggested subsequently that free GCAT contributes only very little to the toxicity of the pathogen because of its tendency to form aggregates with extracellular polysaccharides (Bricknell et al. 1997). The pathogenesis of furunculosis depends on several factors, and GCAT could be only one of these. Moreover, there is evidence that GCAT-knockout mutants do not lose virulence in challenge experiments (Vipond et al. 1998). Nevertheless, the enzyme which was described as cytotoxic to Atlantic salmon (Coleman & Huntly 1994) may be a matter of particular interest in further development of fish vaccination.

The proform of the GCAT enzyme consists of 2 polypeptides connected by a disulfide bridge and it is proteolytically digested during activation (Brumlik et al. 1997). The tendency of activated GCAT to form dimers has been documented (Ausio et al. 1993). The epitope structures of the native enzyme stimulate the immune system to develop protective activities. A humoral immune response has to be directed to the native protein and, additionally, antibodies which inhibit enzymatic activity provide especially good protection.

This paper demonstrates that the enzyme activity of GCAT isolated from *Aeromonas salmonicida* and directed to rainbow trout red blood cells (RBC) can be inhibited by one murine monoclonal antibody under *in vitro* conditions.

For production of GCAT-containing extracellular products (ECPs), *Aeromonas salmonicida* strain MT004 (Ellis et al. 1988) was used and crude ECPs were prepared as described previously (Lachmann et al. 1997). GCAT was purified by anion exchange chromatography on Q Sepharose Fast Flow (QSFF) using a FPLC system (Pharmacia LKB) according to Lachmann et al. (1998).

Ammonium sulphate-precipitated ECP and purified GCAT were separated by SDS-PAGE and GCAT-stained by a substrate reaction as described previously (Lachmann et al. 1997). Western blotting was performed according to Towbin et al. (1979) and immunostaining of GCAT was performed using monoclonal anibodies (Mabs) or a specific rabbit anti-GCAT serum (kindly provided by J. A. Arnesen, Norwegian Institute of Fisheries and Aqualculture, Tromsø, Norway; described by Arnesen et al. 1993). Antibodies bound to GCAT were visualized using horseradish peroxidase-conjugated goat anti-mouse IgG/M and goat anti-rabbit IgG/M antibodies (Dianova).

Esterolytic activity of purified GCAT in solution was estimated spectrophotometrically using 1 mM *p*-nitrophenyl acetate (Sigma) in 0.02 M Tris-buffer, pH 7.0, according to Kok et al. (1993). For estimation of hemolytic activity, (RBCs) from heparinized rainbow trout blood were washed 3 times in PBS (pH 7.4) at 20°C and used in a final concentration of 1% (v/v) in PBS. One hundred µl of purified GCAT in PBS were

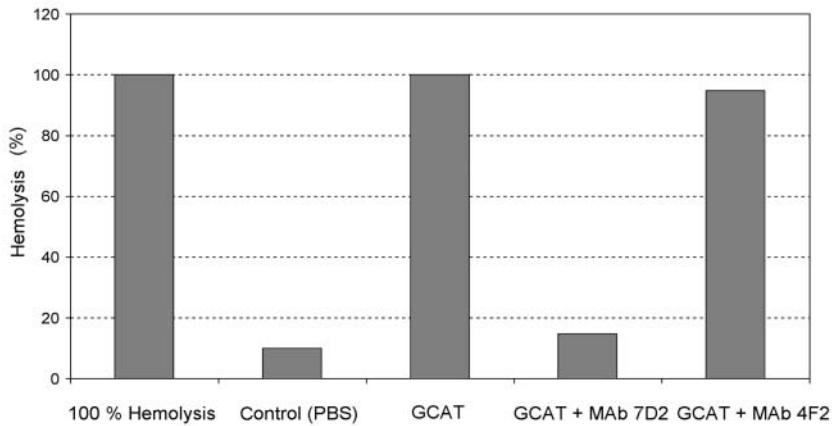---

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Fig. 1. Influence of pre-incubation of 1 µg purified GCAT with anti-GCAT MAb 7D2 and anti-GCAT MAb 4F2 (the anti-GCAT MAbs 7D2, 4G3 and 1D11 showed similar results as 4F2) on the hemolytic activity of the enzyme

added to 1 ml RBC-suspension and incubated at 37°C for 1 h. The hemolytic titres were determined spectro-photometrically as percent of the absorbance at 540 nm of a totally hemolysed sample according to the method of Titball & Munn (1981). Additionally, purified GCAT (500 µg ml^{-1} in PBS) was used for the estimation of the lowest quantities of protein causing 100% hemolysis of RBC.

To analyze inhibition of hemolytic activity by anti-GCAT MAbs, purified GCAT was pre-incubated with an equal volume of hybridoma supernatants contain-ing 1 µg ml^{-1} anti-GCAT MAbs 4F2, 4G3, 1D11 and 7D2, which were described by Lachmann et al. (1998). An irrelevant MAb was used as negative control. The purified GCAT had a strong hemolytic effect on rain-bow trout RBC. Titration showed that total hemolysis of 10^{10} RBC was caused by 10 ng of the purified enzyme. Pre-incubation of 1 µg pure GCAT with anti-GCAT MAbs directed to different native epitopes (Lachmann et al. 1998) showed a total inhibition of hemolysis by MAb 7D2 (Fig. 1). It is conceivable that the epitope structure recognised by the inhibiting MAb 7D2 is sit-uated close to or is otherwise related to the active site of the enzyme. MAb 4F2, 4G3 and 1D11 did not signif-icantly inhibit hemolysis (data not shown) (Fig. 1). The MAbs reacted with native structures of the enzyme but differed in the specific epitopes recognized, as demon-strated by competition studies (Lachmann et al. 1998). MAbs 4F2 and 4G3 bound to the same structure, which seems to be a sub-structure of the epitope of MAb 1D11. It was shown that the epitope of MAb 7D2 has no relation to the epitopes of the other three MAbs. Additionally, these MAbs recognized purified GCAT or GCAT from crude ECP on nitrocellulose only using a native PAGE without SDS under non-reducing con-

ditions for the separation. Both purified GCAT and ECP separation resulted in 1 protein band identified by all 4 MAbs and the rabbit antiserum as GCAT (data not shown) on nitrocellulose. The band arising from ECP seemed to run slower in the native PAGE. SDS-PAGE of GCAT from crude ECP under reduc-ing conditions followed by Western blotting revealed 3 protein bands with molecular weights of approximately 35, 30 and 27 kDa detected by the spe-cific rabbit anti-GCAT serum from Arnesen et al. (1993). These protein bands also showed esterolytic activity. Purified GCAT was detected as the 27 kDa molecule with esterolytic activ-ity. Brumlick & Buckley (1996, 1997) demonstrated trypsin activation of the proform of the enzyme (35.4 kDa) resulting in an excision of a 3.7 kDa peptide. The acti-vated GCAT contains the 27 kDa main polypeptide bound to a smaller peptide of 4.7 kDa. Both are con-nected by a disulfide bridge. A 'nicking mechanism' forms the enzymatically active form of GCAT (31.7 kDa). The 3 bands found with esterolytic activity correspond with findings of Eggset et al. (1994) describing the same properties of various GCAT forms using a protease deficient strain treated with a serine protease. The reactivity of the MAbs, in particular 7D2, with GCAT from crude ECP (Lachmann et al. 1998), and on nitrocellulose after native PAGE, does not clearly exhibit the site of the epitope on the 3 parts of the molecule, but it appears not to be on the 3.7 kDa peptide excised by proteases.

The result of this study suggests that lytic activity of GCAT may be inhibited by a specific MAb but further studies *in vivo* are necessary to corroborate this theory.

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