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NOTE

Generation and preliminary characterisation of monoclonal antibodies directed to glycerophospholipid:cholesterol acyltransferase (GCAT) native epitopes of *Aeromonas salmonicida*

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ABSTRACT: Four monoclonal antibodies (MAbs) directed to native glycerophospholipid:cholesterol acyltransferase (GCAT) epitopes of *Aeromonas salmonicida* were isolated using an esterase capture assay. The molecular mass of this MAb-defined antigen was estimated to be 26 kDa in SDS-PAGE. Three different epitope specificities of these MAbs were demonstrated. It was shown that all 4 MAbs recognize GCAT in culture filtrates of the strain MT004 excluding the simultaneous trapping of other components. None of the MAbs react with the denatured GCAT in Western blots.

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

Glycerophospholipid:cholesterol acyltransferase (GCAT) is a haemolytic active toxin occurring in extracellular products (ECP) of *Aeromonas salmonicida*, the aetiological agent of furunculosis. It appears to be exported as a weakly active 38 kDa proform subsequently activated by an endogenous serine protease and transformed into a highly active 26 kDa form (Eggset et al. 1994). The enzyme has been isolated and purified from culture supernatants and its biochemical properties, including substrate specificity and mechanism of action, have been determined (Buckley 1982, 1983). Lee & Ellis (1989, 1990) demonstrated that a complex of GCAT and lipopolysaccharide (LPS) is the major lethal toxin in ECP of *A. salmonicida*. The results of Bricknell et al. (1997) suggest that GCAT might form a complex with a non-LPS extracellular polysaccharide (EPS), and an antibody response to EPS would protect Atlantic salmon *Salmo salar* L. by neutralising the lethal toxin in its complexed form. The latter authors presented correlations between protective effects and the level of anti-EPS antibodies in salmon. For the development of effective furunculosis vaccines, immunoaccessible epitopes exposed on native virulence molecules of the pathogen are of some interest as targets for protective immune mechanisms. Monoclonal antibodies (MAbs) should be useful for identifying native antigen epitopes which caused a protective immune response in the host and may contribute to the investigation of the antigenic structures of the virulence factor. Thus, they could be appropriate for serological diagnosis, control of infectious fish diseases and for the detection of specific antibody populations potentially protective against GCAT in sera of vaccinated fish. For this purpose, this report demonstrates the generation of MAbs against GCAT of *A. salmonicida* and describes the binding characteristics of these MAbs against native epitopes of the enzyme.

Aeromonas salmonicida strain MT 004 (Ellis et al. 1988) was grown in tryptone soya broth (TSB) (Oxoid, Unipath, Basingstoke, UK) in a shaking water bath for 72 h at 15°C. The ECP were treated with phenylmethylsulfonyl fluoride and then ammonium sulphate precipitated (to 65% saturation). The pellet was fractionated by anion exchange chromatography on Q Sepharose® Fast Flow (QSFF) using step-gradients of NaCl and an FPLC system (Pharmacia LKB, Uppsala, Sweden). To obtain purified and non-denatured GCAT for immunization, a 40% ethanol-containing 0.02 M tris(hydroxy-methyl)amino-methane-HCl buffer, pH 7.7, was employed to disrupt the complexes between LPS or other ECP and GCAT. The enzymatically active GCAT, as determined by its esterolytic activity for *p*-nitrophenyl acetate and its haemolytic activity against rainbow trout red blood cells, could be eluted along a NaCl-gradient at 0.15 M. Following an acetone precipitation of this QSFF-fraction, an esterolytically

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active 26 kDa GCAT was found in non-reducing SDS-PAGE (sodium dodecyl sulphate polyacylamide gel electrophoresis) as described by Lachmann et al. (1997). The GCAT preparation was mixed with GERBU adjuvant (GERBU Biotechnik GmbH, Gaiberg, Germany) and used to immunise mice following gentle heat inactivation as described by Lee & Ellis (1990). Hybridoma generation and antibody production were performed as described by Wagner et al. (1997).

For selection of anti-GCAT MAbs hybridoma supernatants were coated onto ELISA (enzyme-linked immunosorbent assay) plates (Greiner, Frickenhausen, Germany) using goat anti-mouse IgG (Dianova, Hamburg, Germany) and incubated with freshly prepared bacterial supernatant at 4°C. GCAT activity was detected by means of a bound esterolytical activity assay (esterase capture assay) using *p*-nitrophenyl acetate as synthetic substrate (Buckley 1983). Four hybridomas (4F2, 4G3, 7D2, 1D11) producing MAbs which reacted with the native functional enzyme were identified. The specificity of the MAbs for GCAT was tested by incubating the MAb-bound ECP component with rabbit antisera raised against *Aeromonas salmonicida* GCAT (Arnesen et al. 1993), LPS (Bernoth, Berlin) and ECP (Wagner et al. 1997). The bound rabbit antibodies were detected with goat anti-rabbit IgG (Dianova) conjugated with peroxidase. A positive reaction was obtained only with the anti-GCAT serum. This implies that the MAbs only bind free GCAT. It is likely that the detergent Tween 20 may disrupt existing LPS or EPS complexes during the ELISA procedure leaving only free GCAT behind.

Immunoprecipitation experiments (as described by Wagner et al. 1997) were carried out to demonstrate the MAb-bound GCAT. The precipitated antigen was resuspended in SDS sample buffer at room temperature and separated by SDS-PAGE. A 26 kDa protein was detected as an esterolytically active molecule in an azo-dye reaction (Lachmann et al. 1997) (Fig. 1A). The pellet was also boiled in reducing SDS sample buffer, electrophoretically separated, blotted onto a nitrocellulose membrane and immunostained by the rabbit antisera described above. As shown in Fig. 1B the material precipitated reacted only with the rabbit anti-GCAT serum and was identified as the 26 kDa form of GCAT. There was no indication of simultaneous trapping of LPS or other ECP components by the MAbs.

None of the 4 MAbs reacted with the denaturated GCAT in Western blots. This suggests that they recognise conformationally dependant native epitopes. Recently, the *in vivo* expression of several virulence factors of *Aeromonas salmonicida* has been noted by various groups (Røsjø et al. 1993, Bricknell et al. 1997, Ellis et al. 1997). The MAbs should be useful for studying the *in vivo* expression of the cytotoxic GCAT in salmonids.

For epitope mapping by ELISA, the 4 MAbs were biotinylated using 0.5 mg sulpho-N-hydroxy-succinimido-6-(biotinyl-amido)hexanoate (Sigma, St. Louis, MO, USA) per mg immunoglobulin. A competitive binding assay was performed by means of coating ELISA-plates with non-biotinylated MAbs and ECP as described above followed by a blocking solution of 10% horse and 5% mouse normal serum. Then, the MAb-trapped GCAT was incubated with the biotinylated MAbs and the binding of these MAbs was detected by streptavidin biotinylated horseradish peroxidase complexes (Amersham, UK). Mabs 4F2 and

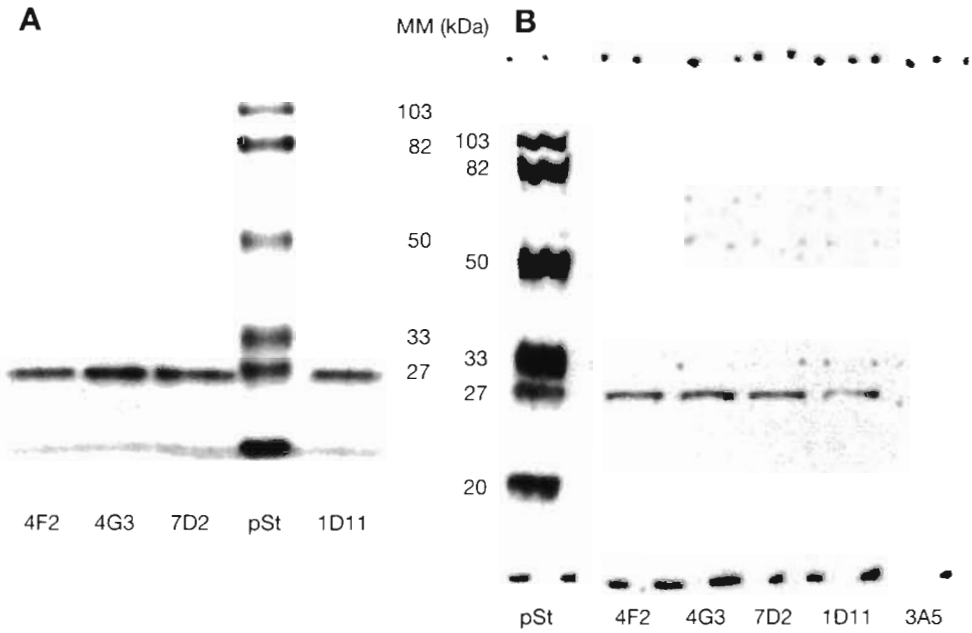

Fig. 1. Specificity of the selected MAbs directed to native GCAT of *Aeromonas salmonicida* strain MT004. The antigen was immunoprecipitated from culture supernatant by anti-GCAT MAbs 4F2, 4G3, 7D2 and 1D11. The irrelevant MAb 3A5 was used as a negative control. (A) SDS-PAGE of immunoprecipitated, SDS-treated and not boiled GCAT detected by its esterolytic activity using the α-naphthyl acetate-azo dye method. (B) Western blot analyses of immunoprecipitated, SDS-treated and boiled GCAT immunostained with a rabbit anti-GCAT serum. Lanes pSt show the prestained molecular mass (MM) standard

4G3 seemed to bind to the same epitope that could also be part of an antigenic structure recognized by MAb 1D11. Mab 7D2 clearly bound to a different epitope. The experiments provided no indication of a recognition of dimeric forms of GCAT in culture supernatants which have been described as a 54 kDa molecule using SDS-PAGE (Lee & Ellis 1990).

It was shown here that the 4 isolated MAbs recognize a native protein in culture supernatants of the pathogen, formerly described as a 27 kDa (Lee & Ellis 1990) or 26 kDa molecule (Eggset et al. 1994), respectively, and known as GCAT. In future research these MAbs will be used for the demonstration of a specific anti-GCAT response in fish after an experimental immunization by means of a sandwich-ELISA.

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