Fish & Shellfish Immunology 29 (2010) 946–955

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Characterisation of an immunodominant, high molecular weight glycoprotein on the surface of infectious *Neoparamoeba* spp., causative agent of amoebic gill disease (AGD) in Atlantic salmon

Margarita Villavedra [a,b], Joyce To [a,b], Susan Lemke [a,b], Debra Birch [d], Phil Crosbie [b,c], Mark Adams [b,c], Kevin Broady [a,b], Barbara Nowak [b,c], Robert L. Raison [a,b], Michael Wallach [a,b,*]

[a] *Institute for the Biotechnology of Infectious Diseases, University of Technology Sydney, PO Box 123, Broadway, NSW 2007, Sydney, Australia*
[b] *Aquafin CRC, Australia*
[c] *National Centre for Marine Conservation and Resource Sustainability, University of Tasmania, Tasmania, Australia*
[d] *Microscopy Unit, Department of Biological Sciences, Macquarie University, NSW, Australia*

ARTICLE INFO

Article history:
Received 24 May 2010
Received in revised form
30 July 2010
Accepted 30 July 2010
Available online 11 August 2010

Keywords:
Neoparamoeba
Amoebic gill disease
Salmon
Glycans
Mucin
Glycoprotein

ABSTRACT

Amoebic gill disease can be experimentally induced by the exposure of salmonids to *Neoparamoeba* spp. freshly isolated from infected fish, while cultured amoebae are non-infective. Results from our previous work suggested that one key difference between infectious and non-infectious *Neoparamoeba* were the highly glycosylated molecules in the glycocalyx. To characterise these surface glycans or glycoproteins we used a monoclonal antibody (mAb 44C12) specific to a surface molecule unique to infective parasites. This mAb recognised a carbohydrate epitope on a high molecular weight antigen (HMWA) that make up 15–19% of the total protein in a soluble extract of infectious parasites. The HMWA consisted of at least four glycoprotein subunits of molecular weight (MW) greater than 150 kDa that form disulfide-linked complexes of MW greater than 600 kDa. Chemical deglycosylation yielded at least four protein bands of approximate MW 46, 34, 28 and 18 kDA. While a similar HMWA complex was present in non-infective parasites, the glycoprotein subunits were of lower MW and exhibited differences in glycosylation. The four glycoproteins subunits recognised by mAb 44C12 were resistant to degradation by PNGase F, PNGase A, O-glycosidase plus β-1, 4-galactosidase, β-N-acetylglucosaminidase and neuraminidase. The major monosaccharides in the HMWA from infectious parasites were rhamnose, fucose, galactose, and mannose while sialic acids were absent. The carbohydrate portion constituted more than 90% of the total weight of the HMWA from infectious *Neoparamoeba* spp. Preliminary results indicate that immunisation of salmon with HMWA does not lead to protection against challenge infection; rather it may even have an immunosuppressive effect.

1. Introduction

Neoparamoeba spp. include a group of amoeba that parasitise the gills of marine-farmed Atlantic salmon (*Salmo salar* L.) in Tasmania producing amoebic gill disease (AGD). For a long period *Neoparamoeba pemaquidensis* was considered as the aetiological agent of AGD but recently two new species, *Neoparamoeba branchiphila* and *Neoparamoeba perurans* were identified as possible agents of AGD [1,2]. All three amoebae have been isolated from AGD affected fish but only *N. perurans* n. sp. has been found to be associated with AGD lesions. These results led the authors to conclude that *N. perurans* is the single aetiological agent of AGD [2].

Neoparamoeba spp. isolated from the gills of AGD affected salmon can be grown on lawns of bacteria on malt-yeast-sea water agar or maintained in seawater liquid cultures. However, naïve salmon do not acquire AGD when exposed to cultured amoebae [3,4]. Experimental infections with *Neoparamoeba* spp. can only be established by cohabitation of salmon with infected fish, or by direct exposure of fish to *Neoparamoeba* spp. trophozoites freshly isolated from fish with AGD There are two possible explanations for this fact. The first and most obvious in light of the work of Young et al. [2] is that neither *N. pemaquidensis* nor *N. branchiphila* are aetiological agents of AGD, and overgrow *N. perurans* in culture. Another explanation is that when the parasites are being cultured *in vitro* important changes occur in the expression of surface molecules that are crucial for the infective capability of the parasite [5].

* Corresponding author. Tel.: +61 (0) 2 9514 4082; fax: +61 (0) 2 9514 8206.
E-mail address: Michael.Wallach@uts.edu.au (M. Wallach).

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doi:10.1016/j.fsi.2010.07.036

Our previous work has shown that one of the main differences between infective and non-infective amoebae was the nature of the glycans and/or glycoproteins in the glycocalyx of the parasite [7]. Furthermore, we had shown that high molecular weight cell-surface glycans are immunodominant in both immunized mice [7] and fish experimentally infected with AGD [6].

In this article we characterise the immunodominant glycoproteins expressed in the glycocalyx of infectious *Neoparamoeba* spp. and assess the vaccine potential of these molecules in an immunisation/challenge trial.

2. Materials and methods

2.1. Amoebae

2.1.1. Non-infectious N. pemaquidensis

N. pemaquidensis, (designated PAO27) originally isolated and cloned from AGD affected salmon was grown on malt-yeast-seawater agar plates as described in Villavedra et al. [5]. This culture was first established in 1994 and is non-infectious.

2.1.2. Infectious Neoparamoeba spp.

Freshly isolated infectious *Neoparamoeba* spp. were obtained according to the method of Morrison et al. [8] from an ongoing infection of co-specific Atlantic salmon using cohabitation maintained at the University of Tasmania. Although this is a mixed assemblage of amoebae species *N. perurans* is overwhelmingly predominant as PCR analyses of the isolates at various times over 2 years showed that only *N. perurans* could be identified and not the other 2 *Neoparamoeba* species [2,9]. This indicates that the other species are either not present or present but in undetectable numbers, however for the purposes of this paper all amoebae isolated from salmon with AGD and used to challenge naive salmon in a subsequent infection trial and other experiments are referred to as infectious *Neoparamoeba* spp.

2.2. Parasite soluble fraction

Approximately 6 million infectious *Neoparamoeba* spp. that were stored frozen at −80 °C, were thawed, centrifuged at 400 g for 15 min at 10 °C, washed in PBS and subjected to osmotic lysis by re-suspension in 250 μL of MilliQ water with complete protease inhibitor cocktail (Roche). The parasite suspension was frozen at −20 °C, thawed at 37 °C and centrifuged at 16,000 g for 15 min at 10 °C, the supernatant was removed and the pellet resuspended as above then subjected to two further freeze/thaw/centrifuge cycles (i.e. a total of 3 cycles). The supernatants were pooled and the protein concentration determined using the BCA Protein Assay Kit prior to freezing (Pierce) and stored at −80 °C.

2.3. Monoclonal antibodies

The hybridoma cell line producing the IgM monoclonal antibody 44C12 was developed along with several others by a subtractive immunisation method and cloned by limiting dilution as described in Villavedra et al. [7]. Briefly, Balb/c mice were injected i.p with sonicated non-infectious *N. pemaquidensis* followed 24 and 48 h later by an i.p. injection of 100 μL of a 20 mg/mL solution of cyclophosphamide monohydrate in PBS. This cycle was repeated 3 times with two week intervals between cycles. This treatment was followed 7–10 days later by positive immunisation with sonicated infectious *Neoparamoeba* spp. parasites emulsified in Freund's Incomplete Adjuvant (FIA). MAb 44C12 was selected because it recognises a carbohydrate epitope on a high molecular weight antigen (HMWA) on the surface of infectious *Neoparamoeba* spp. and does not react with cultured, non-infectious *N. pemaquidensis* [6].

2.4. SDS-PAGE analysis

The parasite soluble fraction protein (4–9 μg/lane) with or without 100 mM dithiothreitol (DTT), were separated on SDS-PAGE 4–12% gradients gels. Proteins were visualised by silver staining and Pro-Q Emerald 300 Glycoprotein Gel and Blot Stain (Molecular Probes) was used for glycoprotein staining.

Native gel electrophoresis was performed using a NuPAGE Novex Tris-acetate gel (Invitrogen) and NuPAGE Tris-acetate running buffer (Invitrogen). High molecular weight markers (HMW calibration kit for native electrophoresis) were from Amersham.

2.5. Immunoblot

Immunoblot was performed as described in Villavedra et al. [7]. Briefly, proteins separated on SDS-PAGE were transferred to nitrocellulose membranes, blocked with 5% skimmed milk, and incubated for 2 h at room temperature with mouse serum or tissue culture supernatant of selected hybridomas. Anti-mouse IgG, γ-chain specific and/or anti− mouse IgM, μ − chain specific alkaline phosphatase conjugates were used as secondary antibodies.

2.6. Indirect immunofluorescent-antibody test (IFAT)

IFAT was performed as described in Villavedra et al. [7]. Briefly, parasites were heat fixed on glass slides and incubated with hybridoma supernatant for 2 h at 37 °C. FITC conjugated anti-mouse IgG, γ-chain specific or anti-mouse IgM, μ chain specific antibodies were used as secondary antibodies.

2.7. Confocal microscopy

Freshly isolated (within 24–48 h after isolation) infectious *Neoparamoeba* spp. were fixed with 2% paraformaldehyde in PBS for 15 min, washed three times with PBS and incubated for 10 min on a slide pre-treated with 0.1% polyethylenimine (PEI). Cells were then permeabilised by incubation with 100 μL of cold methanol for 10 min, washed three times with PBS and blocked with PBS-10% foetal bovine serum for 30 min. One hundred microliters of hybridoma supernatant or purified mAb (10 μg mL^{-1}) were added onto the slides and incubated for 1 h at room temperature in a moist chamber. After washing, an anti-mouse immunoglobulins Alexa fluor 488 conjugate (Molecular probes) was added and the slides incubated for 1 h at room temperature. The slides were washed, mounted in FluorSave Reagent (Calbiochem) and visualised using an Olympus Fluoview 300 confocal microscope. Purified mouse IgMκ (MOPC 104E, Sigma USA) was used as a negative control.

2.8. Transmission electron microscopy (TEM)

Freshly isolated (within 24–48 h after isolation infectious) *Neoparamoeba* spp. (3×10^5 mL^{-1}) were fixed in 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M Pipes buffer (pH 7.2) for 1 h, washed in 0.1 M Pipes, suspended in 1% low melt agarose at 55 °C and spun immediately to obtain a pellet of cells in the agarose. The pellet was cut into two small segments, which were suspended in 0.1 M Pipes buffer. The agarose pellets were dehydrated in a graded series of ethanol, infiltrated and embedded in LR White resin. Ultrathin sections (70 nm) were cut on an ultramicrotome (Reichert Ultracut E) and collected on 300 mesh Pioloform coated grids. Sections were incubated in 0.05 M glycine in PBS for