Fish & Shellfish Immunology 34 (2013) 789–798

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

Immune parameters correlating with reduced susceptibility to pancreas disease in experimentally challenged Atlantic salmon (*Salmo salar*)

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ARTICLE INFO

Article history:
Received 21 December 2011
Received in revised form
23 November 2012
Accepted 10 December 2012
Available online 7 January 2013

Keywords:
Salmonid alphavirus
Disease resistance
Adaptive
Innate
Neutralising antibodies

ABSTRACT

Two strains of Atlantic salmon (*Salmon salar*) with different susceptibility to infectious salmon anaemia (ISA) were challenged with salmon pancreas disease virus (SPDV), the etiological agent of salmon pancreas disease (PD), by cohabitation. Serum and tissues were sampled at 0, 1, 3, 6 and 8 weeks post-challenge. Experimental challenge with SAV did not cause mortality, but virus loads and assessment of histopathology indicated that the fish more resistant to ISAV (ISAHi) also was more resistant to PD. Eight weeks post-challenge, the ISAHi strain had higher titres of SAV-neutralising antibodies than the less resistant strain (ISALo). Transcript levels of four adaptive and six innate immune parameters were analysed by real-time RT-PCR in heart, head kidney (HK) and gills of both strains. Secretory IgM (sIgM) and CD8 levels differed most between the two salmon strains. The ISAHi strain had significantly higher levels of sIgM in HK at all samplings, and significantly higher CD8 levels in gills at most samplings. In heart, both sIgM and CD8 levels increased significantly during the challenge, but the increase appeared earlier for the ISALo strain. By hierarchical clustering analysis of mRNA levels, a clear segregation was observed between the two strains prior to the virus challenge. As the viral infection developed, the clustering divide between fish strains disappeared, first for innate and later for adaptive parameters. At eight weeks post-challenge, the divide had however reformed for adaptive parameters. Possible pair-wise correlation between transcript levels of immune parameters was evaluated by a non-parametric statistical test. For innate parameters, the extent of correlation peaked at 3 wpc in all tissues; this came rapidly for ISALo and more gradual for ISAHi. The ISAHi strain tended to show higher correlation for innate parameters in heart and gill than ISALo at early sampling times. For adaptive immune parameters, little correlation was observed in general, except for ISAHi in heart at 6 wpc.

Overall, the observed differences in immune parameters may provide important clues to the causes underlying the observed difference in susceptibility to PD.

1. Introduction

Salmon pancreas disease (PD) is caused by an alphavirus called *Salmon pancreas disease virus* [1]. Due to its taxonomic placement, the name *Salmonid alphavirus* (SAV) has been proposed and is now commonly used [2]. Different isolates of SAV can only be discriminated by genetic analysis. Based on genotypic variations, it is currently proposed that SAV should be divided into six genogroups (subtypes) [3], each correlating with geographical separation.

Abbreviations: ISAHi, fish strain with relative high resistance to infection with ISAV; ISALo, fish strain with relative high resistance to infection with ISAV.

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http://dx.doi.org/10.1016/j.fsi.2012.12.014

Whereas PD refers to the disease in seawater farmed Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) in the British Isles/Norway, a related disease called sleeping disease (SD) is mainly found in rainbow trout reared in freshwater (Central Europe). SD in trout caused by subtype SAV2 and SPD in the British Isles is most commonly caused by SAV1, while the Norwegian outbreaks of SAV have been restricted to the subtype 3 [4].

PD is an emerging disease in the Atlantic salmon farming industry in Europe. In Norway a significant increase of disease outbreaks in the southern part of the country has occurred since 2003 [5]. In field outbreaks of PD, clinical signs and severity of pathological changes in the key target organs pancreas, heart and skeletal muscle, will vary between individuals [6]. Clinical signs include lethargy and impaired swimming performance; the latter interpreted as the "sleeping" behaviour typical for SD. Depending on temperature and route of infection, infected fish within days develop viraemia that normally lasts for 2 weeks [7,8]. In SAV cohabitant challenge experiments, SAV infection and PD pathology is found from 3 wpc after shedding [9], but generally experimental challenges do not induce mortality. Waning of viraemia has been shown to coincide in time, but not overlap, with the emergence of neutralizing antibodies (NAb) in the bloodstream [10], which may happen as early as 10 days after challenge by intraperitoneal injection [11]. Tissues [7,8] and blood cells [7] become virus positive within few days, and virus may remain in these cellular compartments weeks after virus have been cleared from the serum. During SAV infection, lesions develop in a definite temporal manner, affecting first pancreas and heart followed by skeletal musculature and possibly CNS [6,10]. Atlantic salmon surviving experimental PD develops protective immunity that can last at least nine months [12]. As reoccurrence of PD has not been reported from previously infected field populations, the protection obtained from natural PD is likely long-lasting, at least on population level [6]. This protective immunity is likely conferred by NAb, as indicated by the protection gained in fish receiving injection of serum from PD convalescent fish [13]. Susceptibility to PD has been shown to vary between commercial strains of Atlantic salmon [10], but the underlying reason is not well known. In response to experimental infection, leukocyte phagocytic activity was shown to increase, as were levels of lysozyme and complement [14]. Reduced PD specific pathological lesions and SAV3 levels in experimentally infected Atlantic salmon injected with the toll-like receptor (TLR)-ligands polyI:C and CpG have recently been reported, and it was demonstrated that this correlated with transcriptional up-regulation of IFNa1, IFNγ, Mx and the chemokine CXCL10 [15]. The study found that the IFN system participates in the host defence against SAV, as also indicated by the increased levels of IFNa1 and Mx in SAV challenged salmon [16]. Mammalian alphaviruses are also strong inducers of IFN-I resulting in transcriptional up-regulation of genes with antiviral activities [17]. Studies of alphavirus infection in mice deficient in IFN-I signalling have indicated that this pathway is a primary protective response [17,18]. The viral non-structural protein nsP2 of mammalian alphaviruses is an important regulator of virus–host cell interactions and plays a significant role in suppressing the antiviral response [19]. Experimental vaccines using formalin inactivated [6,20] or attenuated SAV [21] induce protection, but commercial vaccines have not been able to fully control the disease in the field. Description of SAV-specific induction of innate and acquired immune responses, and in particular the nature of protective response may aid the development of efficient prophylaxis.

The possibility that increased resistance to a specific pathogen in a bred fish strain may affect susceptibility to other pathogens has been given little attention. In the present study, two strains of Atlantic salmon with differing susceptibility to ISA were challenged with SAV by cohabitation. Based on morbidity parameters and quantification of virus RNA, the two fish strains were shown to differ in susceptibility to PD. In an attempt to resolve the nature of immune factors underlying this differential disease susceptibility, an array of adaptive and innate immune parameters were analysed by real-time reverse transcription quantitative PCR (RT-qPCR) and by serological methods.

2. Materials and methods

2.1. Fish

Atlantic salmon (*S. salar*) of two different strains were obtained from SalmoBreed AS (Bergen, Norway) and kept at the ILAB facility (Bergen, Norway). One strain (ISAHi) was characterised by relative high resistance to infection with ISAV (estimated breeding value of 26.3%) whereas the other (ISALo) had relative low resistance (estimated breeding value of −18%). A third strain of A. salmon (ILAB/08/004) was obtained from ILAB and used as virus shedder fish in cohabitation challenge. Prior to use, the fish were tested and found negative for presence of infectious pancreas necrosis virus (IPNV) and SAV by real-time RT-PCR. The fish were kept at 10–13 °C and fed ad libitum during the challenge.

2.2. Cohabitation challenge and sampling

For the cohabitation challenge, a Norwegian isolate of SPDV (PD03 13p2) passed twice in CHSE-214 cells, was used to inject the shedder fish. The challenge was performed in two tanks, tank A containing 100 salmon of strain ISAHi (mean weight 35 g) and tank B containing 100 salmon of strain ISALo (mean weight 35 g). At day 0 (0 wpc), each of tanks A and B received 25 shedders of strain ILAB/08/004 that had got an intraperitoneal (ip) injection of 0.2 ml virus suspension the same day. Although the exact timing of infection is not possible to pin-point in a cohabitant challenge model this was partly compensated for by using a relatively large number of shedder fish.

At day 0, prior to infection, blood and tissue (head kidney (HK), heart and gill) were sampled from 10 fish from each of strain ISAHi and ISALo. Blood samples were taken from anaesthetized fish using heparin-containing Vacutainers and serum was prepared by standard centrifugation protocol and stored at −20 °C. Tissue samples were immediately transferred to ice-cold RNAlater (Ambion), then incubated at 4 °C for 24 h and finally stored at −20 °C. During the subsequent challenge, identical samplings were performed at 1, 3, 6 and 8 wpc.

At separate samplings, at 4 and 6 wpc, additional heart tissue samples were taken from 15 fish from each of strain ISAHi and ISALo and fixed in 3.5% formaldehyde in buffered saline (pH 7.0).

2.3. Histopathological examination

Formalin fixed heart tissue was processed for haematoxylin–eosin staining using standard protocol. A score system was used to evaluate the severity of SAV induced heart lesions, i.e. no lesion: minimal: 1, mild: 2, moderate: 3 and severe: 4 [22]. Scores $\geq$2 are considered to be specifically to clinical PD infection. Moribund fish and fish that have died from PD are typically scored as severe (4). The scoring was done as a blinded experiment.

2.4. RNA extraction and cDNA synthesis

Tissue samples in RNAlater were distributed between four contributing laboratories (A–D), where total RNA was isolated by use of RNeasy Mini Kit (Qiagen) and eluted in 50 μl of RNase free H_2O. The RNA output was checked by gel electrophoresis for the