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Gene expression profile of HIRRV G and N protein gene vaccinated Japanese flounder, *Paralichthys olivaceus* during HIRRV infection

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ABSTRACT

The glycoprotein (G protein) gene, but not the nucleocapsid protein (N protein) gene, of the hirame rhabdovirus (HIRRV) was previously shown to be highly effective in inducing a protective immune response in Japanese flounder (*Paralichthys olivaceus*) when used as a DNA vaccine. Our previous cDNA microarray analysis demonstrated that interferon-stimulated genes (ISGs) were strongly induced by the HIRRV G protein gene (pHRV-G) but not by the N protein gene (pHRV-N). However, the molecular basis for the difference in protective immunity between pHRV-G- and pHRV-N-vaccinated fish during HIRRV infection is still unclear. In this study, we use a DNA microarray to analyze differences of gene expression in pHRV-G- and pHRV-N-vaccinated fish during HIRRV infection. Microarray analyses showed substantial difference in gene expression patterns during HIRRV infection between fish vaccinated with pHRV-G and pHRV-N. In addition, genes having homology to mammalian T cell activation-related genes were up-regulated in the HIRRV G protein-vaccinated group.

1. Introduction

DNA vaccines using the glycoprotein (G protein) gene of the fish rhabdoviruses (G-DNAv) are highly effective in inducing a short term, non-specific immune response as well as long term adaptive immunity in fish [1,2]. With regards to long term response, it has been reported that the viral hemorrhagic septicemia virus (VHSV) G-DNAv significantly protected fish against viral challenge after 9 months of post-vaccination [3], and the infectious hematopoietic necrosis virus (IHNV) G-DNAv protected fish after 25 months of post-vaccination [4]. The G-DNAv of fish rhabdoviruses also induce an early non-specific protection [5–8]. On the contrary, DNA vaccines using the nucleocapsid (N) protein gene of VHSV [9], or the N, phospho- (P), non-virion (NV) or matrix (M) protein genes of IHNV were unable to stimulate protective immunity in rainbow trout [10].

In rainbow trout, it has been suggested that salmonid rhabdovirus G-DNAv induce both an early non-specific protection at 1–4 weeks of post-vaccination and a later specific protection [2]. The early non-specific protection by fish rhabdovirus G-DNAv has been characterized as a type I interferon (IFN)-mediated, non-specific protection based upon expression patterns of a suite of type I interferon-responsive genes [8,11–14]. The onset of the specific phase of protection by fish rhabdovirus G-DNAv has been suggested to correlate with the development of detectable neutralizing antibodies [10]. However, despite providing a high level of protection, neutralizing antibodies could not be detected in rainbow trout challenged at 9 months after vaccination with VHSV G-DNAv [3] or challenged at 2 years after vaccination with IHNV G-DNAv [4]. The observations of significant protection without detectable neutralizing antibodies suggest that other, probably cellular, mechanisms are involved in the specific protection by

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fish rhabdovirus G-DNAv. The mechanisms of the specific immune response induced by fish rhabdovirus G-DNAv remain to be described in detail.

Hirame rhabdovirus (HIRRV), a pathogen of Japanese flounder (*Paralichthys olivaceus*), is composed of five structural proteins analogous to those of IHNV and VHSV [15]. The G protein, but not the N protein of HIRRV can induce high protective immunity when used as a DNA vaccine in Japanese flounder [16–18]. We have previously demonstrated that type I IFN-inducible genes were strongly induced at day 7 after injection by the HIRRV G protein gene DNA vaccine (pHRV-G) but not by the HIRRV N protein gene DNA vaccine (pHRV-N) [18]. However, the molecular basis for the difference in protective immunity between pHRV-G- and pHRV-N-vaccinated fish during HIRRV infection is still unclear. In this study, we used a DNA microarray to analyze differences of gene expression in pHRV-G- and pHRV-N-vaccinated fish during HIRRV infection. Microarray analyses showed that gene expression patterns were quite different between the pHRV-G-vaccinated group and the pHRV-N-vaccinated group during active HIRRV infection. In addition, genes with homology to mammalian T cell activation-related genes were up-regulated in the pHRV-G-vaccinated group.

2. Materials and methods

2.1. Propagation of viruses

The HIRRV isolate 8601H was propagated in the hirame natural embryo (HINAE) cell line [19] and the virus titer was calculated as 50% tissue-culture infected dose ($TCID_{50}$) using a 96-well cell culture plate (Corning, USA) as previously described [17].

2.2. Vaccination, challenge and sampling for a microarray experiment

DNA vaccines encoding HIRRV G protein gene (pHRV-G) [17] and N protein gene (pHRV-N) [18] in the expression vector pCI-neo (Promega, USA) were used for this study. Both DNA vaccine constructs and pCI-neo vector were purified from overnight cultures of transformed *Escherichia coli* JM109 strain by ultracentrifugation using a CsCl–ethidium bromide gradient [20].

Summary of the vaccination, challenge and sampling schedule is illustrated in Fig. 1(A). Four groups of 50 flounder juveniles with an average weight of 2 g were prepared for injection with pHRV-G, pHRV-N, pCI-neo vector, or PBS. The concentration of DNA in the vaccines was adjusted to 10 μg/50 μl with PBS. Each fish was intramuscularly injected with 50 μl of DNA or PBS. Each group of fish was kept in a 60-l tank in a flow-through water system supplied with seawater at 15 °C. At 28 days after vaccination, each fish was injected intramuscularly with 3.2×10^3 $TCID_{50}$ of live HIRRV isolate 8601H in 50 μl saline buffer. Mortality was first observed at 2 days after the challenge among fish vaccinated with pHRV-N, pCI-neo vector or PBS, and the mortality rate among these groups reached more than 65% on day 4 (Fig. 1B). Five individual fish were sampled from each of the pHRV-G, pHRV-N and pCI-neo vector groups at 1 and 3 days after experimental challenge with HIRRV for microarray analysis.

2.3. DNA microarray experiments

We constructed a fourth version of the Japanese flounder cDNA microarray, which contained 1946 clones that were obtained from EST libraries [21–26]. The biodefense- and immune-related genes present on this microarray are listed in [27]. External control genes, like fungal DNA, lambda DNA and vector plasmid DNA were also spotted onto each of 16 blocks on the microarray to serve as negative controls. Each gene was spotted in duplicate at different parts of the microarray to assess the consistency of hybridization and facilitate comparison during the analysis.

Microarray analysis was carried out as previously described [28]. Briefly, total RNA was isolated using TRIzol (Invitrogen Life Technologies, USA) from the kidney cells at 1 and 3 days after injection and 25 μg was used for cDNA synthesis using a labelstar array kit (Qiagen, Japan). RNA samples obtained from kidney cells of fish receiving only the pCI-neo vector served as unvaccinated controls. Purified cDNAs were labeled with Cy3 or Cy5. Hybridization was performed for 18 h at 42 °C. After 18 h, the array was washed once in 5× SSC–0.1% SDS for 10 min at 30 °C, twice in 0.5× SSC for 2 min at room temperature, followed by a rinse with 0.5× SSC–0.01% Tween at room temperature. The slides were dried and scanned immediately using a Gene Pix 4000B (Axon Instruments, USA).

Images obtained from scanning were analyzed using a commercial software package (GenePix Pro ver. 4.0, Axon Instruments, USA). Analysis of microarray data was performed as previously described [18]. Briefly, low signal intensity spots (<external control spots + PMT 200) were removed. The signal intensity was calculated as the mean intensity of duplicate features minus the background signal. The feature ratio was calculated from the signal intensity of vaccinated fish samples divided by the signal intensity of control fish samples. Genes with feature ratios over 2.0 were considered as up-regulated and genes with feature ratios less than 0.5 were considered as down-regulated [18]. The significance of differences between the vaccinated and control fish was determined with a paired *t*-test on replicated spots for each gene as previously described [29]. *P* values of less than 0.05 were considered significant. The expression ratio data were imported into Cluster 3.0 [30] in conjunction with a *k*-means clustering algorithm, using Euclidian distance as the similarity metric. After clustering, the results were visualized in tree structure by using TreeView program [31].

3. Results

3.1. Differentially regulated genes

The Japanese flounder cDNA microarrays used in this study consisted of 1956 distinct genes. Compared to the expression levels of pCI-neo vector controls, the highest