SHORT COMMUNICATION

Immunostimulation of juvenile turbot (Scophthalmus maximus L.) using an alginate with high mannuronic acid content administered via the live food organism Artemia

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in the present experiments we have demonstrated increased protection against a pathogenic bacterium in juvenile turbot (Scophthalmus maximus L.), after feeding the juveniles the live food organism Artemia enriched with a suspension of alginate micro-spheres containing an immunostimulant. Most likely the effect was due to stimulation of the non-specific defence of the juveniles.

Poor control of the microbial conditions in the intensive rearing facilities may adversely affect the health of fish larvae, and is most likely one of the main factors restricting production of marine juveniles. Methods which help to control the micro-flora in the system or improve the resistance of the larvae against bacterial infection may substantially improve larval viability (Vadstein et al., 1995). Newly hatched marine fish larvae have not developed a specific immune defence. Except for eventual maternally transferred immunity, as described for tilapias (Mor & Avtalion, 1990), the larvae therefore strongly depend on non-specific immunity against bacterial infections. Several studies have shown that the non-specific defence system of fish can be stimulated (Robertsen et al., 1991; Anderson, 1992). Alginate is reported to have a strong stimulatory effect on cytokine production by human monocytes, with the mannuronic acid as the major active component (Otterlei et al., 1991; Espenik et al., 1993). Incubation of yolk sac larvae of Atlantic halibut (Hippoglossus hippoglossus L.) in water to which mannuronic acid rich alginate (FMI) was added, improved the survival rate (Vadstein et al., 1993). This suggests that FMI may stimulate the non-specific system of marine fish larvae.

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In the present study attempts were made to stimulate the macrophage system of turbot juveniles, by feeding them Artemia nauplii enriched with alginate micro-spheres containing FMI. To avoid the period of high mortality usually associated with the early larval stages of turbot (Reitan et al., 1990), 40 days old turbot were used. Immunostimulation was evaluated in an experiment in vitro by monitoring mortality rates after a challenge to Vibrio anguillarum (Chair et al., 1994).

Juvenile turbot were purchased from the hatchery ‘France Turbot’, Noirmoutier, FRANCE. The experiment was designed as a 2 x 2 factorial experiment with five replicates of 25 fishes for each treatment: (1) control (C); (2) immunostimulated (I); (3) challenged control (CC); and (4) challenged immunostimulated fish (CI). The fish were transferred to aquaria of 3 l for acclimation two days before the immunostimulation. The water was aerated and one fifth was renewed once every day by siphoning. The water temperature was kept at 19–20°C.

Alginate rich in mannuronic acid (designated FMI) was isolated from the fruiting bodies of a marine alga, Nostocspora nodosum as described by Haug et al. (1987). The material contained 86% mannuronic acid and had a molecular weight (MW) >300 000. The composition was analyzed by 'H-nmr spectroscopy on a Bruker WM 400 spectrometer as described by Grasladel (1985). The molecular weight was estimated from intrinsic viscosity measurements in an automatic dilution viscosity system (Scott Gerste) using the Mark Houwink-Sakurada equation (Martinsen et al., 1991).

Micro-spheres containing FMI were prepared by entrapping the polymer in a calcium alginate gel. Because FMI is a poor gel former due to the lack of long guluronic acid blocks (Smidsrod & Skjåk-Bræk, 1990), the polymer was mixed with a commercial sample of sodium alginate LF 10/60 with a high content of guluronic acid (GuA=70%; MW=220 000) isolated from Laminaria hyperborea stipes (Pronova Biopolymers, Drammen, Norway). FMI (1 g) and LF 10/60 (3 g) were dissolved in 360 ml of distilled water. The viscous solution was sprayed through a nozzle into an aqueous solution of calcium chloride (50 mM) and methanol (5%). The alginate gel-spheres were washed on a sieve with 500 ml of an aqueous solution containing 3% CaCl2 and finally suspended in 200 ml of the washing solution. The diameter of the micro-spheres was in the range 2-30 μm, with 85% between 2.5 and 5 μm.

The micro-spheres were washed in distilled water and centrifuged (5000 rpm, 2 min) twice, and the waste water drained off before they were added to cultures of 12-24 h-starved Artemia at concentrations of 1 g (wet weight) micro-spheres per 100 ml culture. The Artemia were enriched for 2 h at densities of 250-300 individuals ml⁻¹ and 26-28°C with active aeration, whereafter the water was exchanged and the animals were fed to the groups of turbot to be immunostimulated (I- and CI-groups). The turbot were given the FMI-enriched Artemia in four small rations over a period of 24 h; in total 700 nauplii per fish. The control fish received four corresponding meals of ordinary Super Selco-enriched Artemia.

The live food organism Artemia was prepared by adding 2-3 g dry cysts (GSLE-EG grade, INVE Aquaculture, Bassrode, Belgium) to a glass cone with 11 sea water at 28°C with active aeration. After 24 h the hatched nauplii were separated from the unhatched cysts and cyst-shells, siphoned onto a 125 μm plankton net and washed carefully with seawater (20°C). The nauplii were transferred to cones with 28°C seawater and active aeration in densities of 2-300 individuals ml⁻¹. Rations of 0.3 g Super Selco (INVE Aquaculture) 1⁻¹ were added to the cultures for enrichment of the nauplii (Løger et al., 1989). The turbot were fed daily with the Artemia which had been enriched twice with 0.3 g during 24 h. The nauplii were fed to the fish twice a day, in densities of 300-500 nauplii fish⁻¹ day⁻¹.

Two days after the first ration of FMI-enriched Artemia was offered to the fish, the juvenile turbot were challenged by exposure to V. anguillarum. The four groups of fish were transferred to four 3 l aquaria. V. anguillarum isolated from a disease outbreak in adult sea bass Dicentrarchus labrax (purchased from the Department of Biological Sciences, Heriot Watt University, Edinburgh, Scotland) was cultured for 24 h on Marine agar (Difco) at 25°C. For the two groups to be challenged (CC- and CI-groups) V. anguillarum was added to the water in concentrations of 10⁶ CFU ml⁻¹. After 30
The turbot cleared the water of Artemia nauplii between each feeding. The total mortality of the turbot in the four treatments one week after the challenge is presented in Fig. 1. The mortality was low and not significantly different in the C- and I-groups (P=0.15, Wilcoxon’s test). The challenge entailed moderate mortality rates, with an average mortality of 37.2 ± 1.3% (S.E.M.) in the CC-group and 19.2 ± 4.7% in the CI-group. The difference was highly statistically significant (P=0.005), and showed that feeding the immunostimulant to the turbot gave protection against V. anguillarum and reduced the mortality of the fish by 48%. In a preliminary experiment, terminated at day 3 after the challenge test due to technical problems, the average mortalities were 40.7 ± 14.0 and 25.0 ± 4.6% for the CC- and CI-groups, respectively. This corresponded to an average reduction in the mortality of 39% in the immunostimulated fish.

Low mortality in the C- and I-groups and high average mortality with low variation in the CI-group indicate that the challenge test worked satisfactorily. Assuming that the variance observed for all groups except the CI-group is representative for this type of experiment, significant differences between replicates are suggested for the CI-treatment. These differences may be attributed to the overall methods used for the administration of the immunostimulant, including the enrichment step of the Artemia nauplii with FMI and the regime for feeding the nauplii to the fish. The doses of the immunostimulant ingested by the fish may therefore have been variable. For human monocytes the cytokine stimulating potential of FMI is strongly dose dependent at low concentrations, whereas for higher concentrations there is a wide range giving optimal effect without being toxic (Espevik et al., 1993). The actual dose of accumulated FMI may have varied between fishes, which resulted in variation in the level of immunoprotection against the V. anguillarum. This may explain the tank to tank variation in the immunoprotection. It can be concluded that feeding the immunostimulant to the turbot juvenile nearly halved the mortality of the turbot after challenge with V. anguillarum, and that FMI is a potent immunostimulant for marine fish fry. We do not have results that can provide the average dose of FMI received by the fish.
In this experiment we have demonstrated how the immunostimulant FMI was administered to marine fish larvae through live food, and that it gave protection against a pathogen. Stimulation treatment prior to expected stress events may reduce the larval mortality, and because marine fish larvae are very small and sensitive to handling stress, it would be preferable to administer immunostimulants through the feed. Further examination of the potential of immunotherapy in the farming of marine fish is therefore required. This includes its use at the incubation of yolk sac larvae, during first feeding with live food animals, and during the ongrowing period.

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