***In vitro* immunostimulation of turbot (*Scophthalmus maximus*) leucocytes with β glucan and/or *Photobacterium damsela* bacterin**

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Respiratory burst and phagocytic activities of turbot (*Scophthalmus maximus*) leucocytes were determined after an *in vitro* incubation of head kidneys with different doses of yeast glucan and *Photobacterium damsela* (formerly *Vibrio damsela*) O-antigen. Although there was an increment of these immune functions with the treatment of glucans and O-antigen compared with controls, they were inhibited at high doses (200 $\mu\text{g/ml}$). The optimum dose of glucan was 50 $\mu\text{g/ml}$ when it was administered alone, but a concentration of 10 $\mu\text{g/ml}$ was enough to produce the stimulatory effect.

Key words: glucan, turbot, *Scophthalmus maximus*, immunostimulant, *Photobacterium damsela*, *Vibrio damsela*, immune response

The use of β -1, 3 glucans has been shown to increase the resistance of fish such as carp *Cyprinus carpio* and salmonids against several bacterial infections (Yano *et al.*, 1989; Robertsen *et al.*, 1990; Nikl *et al.*, 1991). This has been attributed to several causes: macrophage stimulation, increment in phagocytic activity of the kidney phagocytes, activation of the alternative pathway of the complement and increment in lysozyme activity. However, the immunostimulant effect varies depending on a number of factors such as the type of glucan used, the administration route, the doses, the administration time, the species, the age, the combination with the vaccine, the vaccine doses etc.

Although β glucans are accepted as potential immunostimulants and adjuvants in fish aquaculture and could be useful tools for general prophylaxis and vaccination, few dosage studies have been carried out. In fact, several authors have described suppression of the fish immune response when a high concentration of immunostimulants was used (Olivier *et al.*, 1985; Yano *et al.*, 1989; Robertsen *et al.*, 1990). Gialdroni and Grassi (1985) established that the immune response at several immunostimulants doses was not linear, meaning that although mean doses could have an enhancing effect over the immune system, slightly high doses could dramatically induce an immunosuppression (Robertsen *et al.*, 1994).

In the present work we establish *in vitro* the optimum yeast glucan doses administered with *Photobacterium damsela* (formerly *Vibrio damsela*) O-antigen for the stimulation of turbot (*Scophthalmus maximus*) leucocytes in order to determine the possible adjuvant effect of this immunostimulant. *Photobacterium damsela* has been recently described causing mortalities in turbot farms in Spain (Fouz *et al.*, 1992), a fish species increasing in importance in European marine aquaculture.

Materials and Methods

Antigens

Photobacterium damsela (strain RG 191) isolated from turbot (Fouz *et al.*, 1992) was plated on Tryptic Soy Agar (TSA, Difco) supplemented with 1.5% NaCl. The bacteria were harvested from trypticase soy broth after 24 h growth at 25°C and processed to obtain O-antigen preparations by the alcohol-acetone extraction (Edwards and Ewing, 1972).

Glucan

The glucan used was a β -1,3 glucan extracted from the cell wall of the yeast *Saccharomyces cerevisiae* (Sigma Chemical Co., USA). Glucan particles were resuspended in PBS (pH 7) (1 mg/ml) and sonicated for one min in a sonicator (Branson).

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Experimental design

The experiments were conducted following Jeney and Anderson (1993) with slight modifications. In brief, head kidneys from 40 turbot (mean weight 100 g) were aseptically removed and placed individually in Petri dishes with 10 ml of Minimum Essential Medium supplemented with 10% of foetal calf serum, 50 IU/ml of penicillin and 50 µg/ml of streptomycin (MEM10). Eight treatments were administered to groups of 5 kidneys: in three groups yeast glucans were added to obtain concentrations of 10 µg/ml, 50 µg/ml and 200 µg/ml, respectively, and 100 µg of a *P. damsela* O-antigen suspension (1 mg/ml). Other three groups were incubated with yeast glucan at concentrations of 10 µg/ml, 50 µg/ml and 200 µg/ml, respectively without the O-antigen. One group was only treated with 100 µg of O-antigen in MEM10. The last group was incubated with PBS. Half of the amount of the medium (5 ml) was replaced daily with MEM10 (with or without glucan). After a 48 h incubation at 15°C the superoxide anion (O_2^-) production and the phagocytic activities were assayed.

Isolation of leucocytes from head kidney

Head kidney leucocytes were isolated according to Santarém *et al.* (1997). Briefly, cell suspensions from head kidney were obtained by teasing the head kidney tissues with forceps in MEM10 supplemented with 10 IU/ml of heparine in a Petri dish. After sedimentation of tissue debris in ice for 10 min, the supernatants were removed and washed twice in the above medium at 300 × g for 10 min at 4°C. Two ml of the cell suspension were layered over discontinuous Percoll density gradient (35-60%) (Pharmacia). After centrifugation at 400 × g for 30 min, the cells at the interface were harvested, washed three times, and suspended in the above medium at a concentration of 1×10^7 leucocytes/ml (containing mainly macrophages and also neutrophils and lymphocytes). Cell viability (95%) was determined by trypan blue exclusion.

Intracellular superoxide anion levels

This test allows to indirectly assess the intracellular O_2^- levels. NitroBlue Tetrazolium (NBT) is reduced by O_2^- produced by phagocytes during the respiratory burst giving a bluish color. The assay of Pick and Mizel (1981) was used as a reference. Briefly, head kidney leucocytes (10^7 cells/ml) were added to a microtiter plate (100 µl per well) and 100 µl of PBS containing 1 mg/ml of NBT (Sigma) was added to each well. Phorbol-12-

myristate-13, acetate (PMA) (Sigma) in a proportion of 1 µl/ml of NBT solution was used to trigger the respiratory burst activity of phagocytes. After 30 min of incubation at 18°C, two washes with PBS were done, centrifuging the plate each time at 600 × g for 3 min. Supernatants were then discharged and cells were solubilized by adding 120 µl of KOH and 140 µl of DMSO (Secombes, 1990). Optical density (OD) was measured at 620 nm in a multiscan spectrophotometer (Labsystems). KOH/DMSO was used as control. Results are given as OD by 10^5 leucocytes.

Phagocytic activity

The phagocytic activity was assayed using the technique described by Dexiang and Ainsworth (1991) with slight modifications. In brief, 100 µl of an anterior head kidney leucocyte suspension (10^7 cells/ml) were mixed with 100 µl of a *P. damsela* bacterial suspension (10^7 cells/ml) in a 96 flat bottom microtiter plate. Following an incubation at room temperature (22°C) for 30 min, the microtiter plates were centrifuged at 150 × g for 2 min and 2/3 of the supernatant were discharged. Smears were made with 10 µl of each well, which were subsequently stained with May-Grunwald Giemsa following standard protocols. This activity is presented as the phagocytic rate [(Number of phagocytes with bacteria/Number of counted phagocytes, usually 200) × 100] and as the phagocytic index [Total number of bacteria ingested by phagocytes/Total number of counted phagocytes (usually 100)].

Statistical analysis.

Data were analyzed by Student's t test. Results are expressed as mean ± standard deviation (S.D.). Differences were considered statistically significant at $p < 0.05$.

Results

Intracellular superoxide anion levels

Respiratory burst activity of turbot leucocytes was enhanced by the *in vitro* administration of different doses of β glucans (10, 50 and 200 µg/ml), alone or combined with *P. damsela* O-antigen (Fig. 1). However, no differences were found between O_2^- levels from kidney leucocytes treated with O-antigen and those from controls (no treatment).

The results of the *in vitro* administration of glucans alone indicate that 50 µg/ml was the best concentration to stimulate the respiratory burst, while with 200 µg/ml,

Fig. 1. NBT reduction by turbot leucocytes from kidneys stimulated *in vitro* with different doses of β -glucans with or without the addition of *Photobacterium damsela* bacterin. Results are expressed as mean ($\pm$ S.D.) of 5 fish. *: Significant differences among the experimental groups and saline control (without O-antigen and without glucan) ($p < 0.01$). *: Significant differences ($p < 0.01$) from controls treated only with O-antigen. †: Significant different from leucocytes treated with 200 $\mu\text{g/ml}$ of glucan ($p < 0.05$). ‡: Significant differences between the O.D. of leucocytes treated with 10 $\mu\text{g/ml}$ of glucan and those treated with 10 $\mu\text{g/ml}$ of glucan and O-antigen.

a significant reduction was observed ($p < 0.05$).

In the case of the combination of glucans and O-antigen, the treatment with glucan significantly increased the O_2^- levels but with higher doses (50 and 200 $\mu\text{g/ml}$) the stimulation tended to be lower. Only when the glucans concentration was 10 $\mu\text{g/ml}$, a significant difference was observed between kidneys treated with or without O-antigen ($p < 0.01$).

Phagocytic activity

The phagocytic rate was significantly higher when glucans were used at concentrations of 10 and 50 $\mu\text{g/ml}$, alone or combined with the O-antigen, compared with the controls (PBS incubated leucocytes) (Fig. 2). At these doses no significant differences were found among the results obtained from the kidneys treated only with glucan or with the combination O-antigen/glucan.

Fig. 2. Phagocytic rate of leucocytes from turbot head kidneys incubated *in vitro* with different doses of β -glucans with or without the addition of *Photobacterium damsela* bacterin. Results are expressed as mean ($\pm$ S.D.) of 5 fish. *: Significant differences among the experimental groups and saline control ($p < 0.01$). *: Significant differences with leucocytes treated with 200 $\mu\text{g/ml}$ and O-antigen ($p < 0.05$).

However, the administration of 200 $\mu\text{g/ml}$ glucan plus 100 μg of O-antigen induced a significant reduction ($p < 0.05$) in the phagocytic rate compared with that obtained with treatments of 10 and 50 $\mu\text{g/ml}$ of glucans, almost reaching the percentage of phagocytosis found in unstimulated head kidney leucocytes (62.1% for leucocytes treated with 200 $\mu\text{g/ml}$ of glucans plus O-antigen and 58.9% for controls).

Phagocytic index represents the ingestion ability of phagocytes and it is shown in Fig. 3. This activity was enhanced when leucocytes were incubated with 10 and 50 $\mu\text{g/ml}$ of glucan with or without O-antigen, compared with controls, although there was a high variability in the results obtained with the different glucan treatments. No differences were found when the results of all the treatments with glucan were compared with those obtained with O-antigen. However, a significant reduction of the phagocytic index was observed with the treatment of 200 μg of glucan plus 100 μg O-antigen, being the phagocytic index even lower than that of unstimulated leucocytes.

Fig. 3. Phagocytic index of leucocytes from turbot head kidneys incubated *in vitro* with different doses of β -glucans with or without the addition of *Photobacterium damsela* bacterin. Results are expressed as mean ($\pm$ S.D.) of 5 fish. *: Significantly different from leucocytes treated with 10 or 50 μ g/ml of glucan alone or combined with O-antigen.

Discussion

Usually, immunostimulants do not show a linear relation between doses and effect but a maximum effect at intermediate doses and no effects and even toxicity at high doses (Bliznakov and Adler, 1972; Gialdroni-Grassi and Grassi, 1985). This has been established in fish *in vivo* (Olivier *et al.*, 1985; Yano *et al.*, 1989, 1991; Robertsen *et al.*, 1990; Anderson, 1992) and *in vitro* (Siwicki *et al.*, 1990; Robertsen *et al.*, 1994). These authors observed an increment in the mortality and suppression of the non-specific immune mechanisms at high doses of immunostimulants such as M-glucan (Robertsen *et al.*, 1990), schizophyllan (Yano *et al.*, 1989), levamisole (Anderson, 1992) and Freund adjuvant (Olivier *et al.*, 1985).

Our results confirm these observations since leucocyte respiratory burst and phagocytic activity were inhibited by the addition of 200 μ g/ml of glucan when compared with lower concentrations. This has been explained in mammals with an ample and rather speculative variety of arguments that go from the receptor competition, the excessive accumulation of immunocompetent cells or

as a consequence of a homeostatic process. Robertsen *et al.* (1990) suggested that in Atlantic salmon (*Salmo salar*) this could be a result of an excess of glucan particles in the phagocytes resulting in an inhibition of the phagocytosis for 1 to 2 weeks until the glucan particles are digested. The *in vitro* studies suggested that this suppression of the phagocyte activity may be a result of the synthesis of inhibition factors secreted by the same cell population or even by the glucan intrinsic toxicity. We have recently shown that macrophage respiratory burst activity can be modulated negatively by addition of exogenous substances such as prostaglandins (Novoa *et al.*, 1996).

It is then obvious that, prior to the use of an immunostimulant on its own or as an adjuvant combined with a vaccine, these doses should be optimized. In fact, Siwicki *et al.* (1990) observed that high doses of levamisole combined with *Yersinia ruckeri* O-antigen caused immunosuppression at the non-specific and specific level. In our case, the glucan optimum doses are different when glucans are used alone or when they are combined with an O-antigen. While the maximum O_2^- levels and phagocytic activity was found with 50 μ g/ml of glucan alone, 10 μ g/ml of glucan combined with 100 μ g of O-antigen were enough to produce the stimulatory effect. It must be pointed out that with 200 μ g of glucan combined with O-antigen, the phagocytic index is even lower than that of the untreated controls.

The fact that the most efficient glucan doses as immunostimulant on its own is higher than that needed when it is combined with the O-antigen could be attributed to a synergic effect (Siwicki *et al.*, 1990), since O-antigens stimulate the non-specific immune mechanisms on their own (Santarém and Figueras, 1994, 1995). Other *in vitro* studies have demonstrated that some molecules capable of up-regulating fish macrophage activity can act synergically (Hardie *et al.*, 1994; Novoa *et al.*, 1996). More studies are needed to investigate combinations of vaccines and immunostimulants to achieve optimum treatment for aquaculture practice.

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