Modulation by extracts of the scuticociliate Philasterides dicentrachi of turbot leucocyte functions and inflammatory cytokine gene expression.

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Abstract: Philasterides dicentrachi is a ciliate causing scuticociliatosis in farmed turbot. This study shows that excretion-secretion products (ESP) and a total ciliate lysate (TCL) of this parasite have different effects on the responses of thioglycollate-induced turbot peritoneal leucocytes. Specifically, leucocytes incubated in vitro with ESP showed increased chemotaxis and increased NO production, while leucocytes incubated in vitro with TCL showed increased phagocytic activity, increased production of reactive oxygen species (ROS), but reduced NO production. Intracellular levels of mRNAs of the pro-inflammatory cytokines interleukin 1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α) peaked about 3 days after inoculation with thioglycollate, while mRNA levels of the anti-inflammatory cytokine transforming growth factor beta (TGF-β) peaked within the first 24 hours. Inoculation of turbot with 3% thioglycollate plus ESP led to increased intraleucocyte mRNA levels of IL-1β, while inoculation with 3% thioglycollate plus TCL led to increased mRNA levels of TGF-β. These results indicate that live and dead Philasterides dicentrachi have various effects on leucocyte function and on the expression of immunoregulatory genes. It seems likely that these immunomodulatory effects will have important consequences for the inflammatory response generated during natural infection of turbot with this parasite.

Keywords: turbot, Philasterides dicentrachi, ciliate, leucocytes, respiratory burst, proinflammatory cytokines

Resumen: Philasterides dicentrachi es un ciliado que provoca la scuticociliatosis en el rodaballo en cultivo. Este estudio demuestra que los productos de excreción-secreción (ESP) y un lisado total del ciliado (TCL) de este parásito produce diferentes efectos sobre la respuesta de leucocitos peritoneales de rodaballoos inducidos con tioglicolato. Específicamente, los leucocitos incubados in vitro con ESP mostraron un incremento en la quimiotaxis y en la producción de óxido nítrico (NO), mientras que los leucocitos incubados in vitro con TCL presentaron un incremento en la actividad fagocítica y en la producción de especies reactivas del oxígeno (ROS), pero una reducción en la producción de NO. Los niveles intracelulares de ARNm de las citocinas proinflamatorias interleucina 1 beta (IL-1β) y el factor de necrosis tumoral alfa (TNF-α) presentaron un pico de expresión al tercer día tras la inoculación con tioglicolato, mientras que los niveles de ARNm de la citocina factor transformante del crecimiento beta (TGF-β) alcanzó la máxima producción alrededor de las primeras 24 horas. La inoculación de rodaballitos con tioglicolato junto con ESP incrementó los niveles de ARNm de IL-1β, mientras que la inoculación con tioglicolato y TCL incrementó los niveles de TGF-β. Estos resultados indican que según el estado de viabilidad P. dicentrachi puede inducir diferentes efectos sobre la función leucocitaria y sobre la expresión de los genes inmunoreguladores. Parece probable que estos efectos inmunomoduladores produzcan importantes consecuencias sobre la respuesta inflamatoria generada durante la infección natural del rodaballo por este parásito.

Palabras clave: rodaballo, Philasterides dicentrachi, ciliado, leucocitos, estrés oxidativo, citocinas proinflamatorias.

1. Introduction
The euryhaline histiophagous ciliate Philasterides dicentrachi was initially described as an opportunistic parasite causing systemic infections in Dicentrarchus labrax in Mediterranean coastal lagoons (Dragesco et al., 1995), but recently it has been recognized as an emergent infectious agent causing important economic losses in farmed turbot, Scophthalmus maximus, on the Atlantic
coast of northwest Spain (Iglesias et al., 2001; Iglesias et al., 2002). *P. dicentrarchi* causes severe systemic disease in turbot, acting as an endoparasite that divides rapidly in many organs and feeds on cells (mainly erythrocytes) and other tissue components, causing the death of the host (Iglesias et al., 2003b). The presence of ciliates in internal organs is associated with intense oedematous inflammation due to accumulation of inflammatory cellular infiltrate (principally monocytes and lymphocytes), especially in the areas showing the highest densities of *P. dicentrarchi* trophozoites (Iglesias et al., 2001).

Phagocytosis is the primordial defense mechanism of all metazoan organisms, including teleosts, to infectious organisms such as parasites (Neuman et al., 2001). Phagocytic cells act in phases: chemotaxis, attachment to the parasite, ingestion, killing and digestion (Verhoef, 1991). Killing of parasites by phagocytes may be oxygen-dependent (respiratory burst) or oxygen-independent (Verhoef, 1991). The respiratory burst, induced by chemotactic stimulation or phagocytosis, involves release of reactive oxygen intermediates (ROS) that kill parasites, but that may also cause tissue damage and inflammation (Baggioni & Wymann, 1990). ROS are potent inducers of the pro-inflammatory stress response typified by pro-inflammatory cytokines, prostaglandins, thromboxanes, leukotrienes, leukocyte adhesion molecules and chemokine synthesis (Tse et al., 2004).

Although very limited information is available on the effect of scuticociliate infections on the fish non-specific immune system, recently we have demonstrated that infection of turbot with the ciliate *P. dicentrarchi* has the following effects on phagocytic cells: (a) modulation of complement activation and intracellular ROS production, (b) slightly increased NO production, and (c) increased scavenging of extracellular ROS (Leiro et al., 2004).

At present, there is no precise information of the modulatory effects of *P. dicentrarchi* extracts on phagocyte function or inflammatory responses in turbot. The aim of the present study was to investigate the modulatory effects of two *P. dicentrarchi* components, a total ciliate lysate (TCL) and excretion-secretion products (ESP), on the main phagocytic functions (namely chemotaxis, phagocytosis, and the respiratory burst) and the expression of genes related to the main cytokines involved in the inflammatory process (interleukin 1 beta, IL-1β; tumour necrosis factor alpha, TNF-α; and transforming growth factor beta, TGF-β).

## 2. Materials and Methods

### 2.1. Fish

Juvenile turbot *Scophthalmus maximus* L. (50-100 g) were obtained from a local farm in the north of Galicia (north-western Spain). Prior to experiments, the fish were acclimatized for at least 15 days in 10-l tanks with a constant flow of water (18 ± 1°C, pH 6.5 ± 0.5) and aeration. The fish received a standard semi-dried pelleted food daily. Blood samples were collected by caudal vein puncture from fish anaesthetized with 0.03% 2-phenoxy ethanol (Sigma). The serum was separated by centrifugation at 2000 x g for 10 min, and stored at -30°C until use.

### 2.2. Parasites and culture

The ciliate *Philasterides dicentrarchi* (Iglesias et al., 2001) was obtained from ascitic fluid of naturally infected turbot, and maintained axenically at 18°C in ‘complete’ L-15 medium (Leibovitz, 10‰, salinity, pH 7.2; Iglesias et al., 2003a), containing 90 mg/l each of adenosine, cytidine and uridine, 150 mg/l of guanosine, 5 g/l of glucose, 400 mg/l of L-α-phosphatidylcholine, 200 mg/l of Tween 80, 10% heat-inactivated foetal bovine serum (FBS) and 10 ml/l of 100 X antibiotic antimycotic solution (= 100 units/ml of penicillin G, 0.1 mg/ml of streptomycin sulphate and 0.25 mg/ml of amphotericin B) (all from Sigma-Aldrich, USA).

### 2.3. Stimuli, drugs and chemicals

Total ciliate lysate (TCL) was obtained as previously described (Iglesias et al., 2003b). Briefly, ciliates collected by centrifugation at 650 x g for 5 min were resuspended in saline phosphate buffer (PBS; 0.015 M phosphate buffer, 0.15 M NaCl, pH 7.2) containing 0.1 mM pepstatin A, 0.02 mM N-(trans-epoxysuccinyl)-L-leucine 4 guanadinobutylamide (E-64), 1 mM phenylmethanesulfonyl fluoride (PMSF) and 2 mM ethylenediaminetetraacetic acid (EDTA) as protease inhibitors (all from Sigma-Aldrich). Subsequently, the ciliates contained in the suspension were lysed ultrasonically in a Branson W-250 sonifier (Branson Ultrasonic Corporation, USA). The whole process was performed on ice. The resulting lysate (TCL) was stored at -80°C.

Excretion-secretion products (ESP) were obtained as previously described (Paramá et al., 2004). Briefly, 2 x 10⁶ cells/ml where incubated for 24 h at 18°C in ‘incomplete’ L-15 medium (like ‘complete’ L-15 medium but without nucleosides, glucose, lipids and FBS). The suspension was filtered through 0.22 μm sterile filters (Iwaki, Japan) and the filtrate concentrated 25 times using YM-10 Microcon centrifugal filter devices (Millipore, USA). The resulting concentrate (ESP) was stored at -80°C until use. Protein concentration in TCL and ESP was determined by Bradford’s method (1976) using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Germany), with bovine serum albumin (Sigma-Aldrich) as standard.
Blood samples were collected by caudal vein puncture from fish anaesthetized with 0.03% 2-phenoxy ethanol (Sigma), and the serum was separated by centrifugation at 2000 x g for 10 min, and stored at -30ºC until use.

*Kluyveromyces lactis* (strain NRRL-41140) cells were cultured on 1% yeast extract, 2% peptone, 2% glucose (YPD medium; Difco, USA) at 30ºC, at a growth rate of 0.07/h, then washed with distilled water and finally freeze-dried. Stock solution (2 mg/ml) of phorbol 12-myristate 13-acetate (PMA) (Sigma, St Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO) and stored in the dark at -80ºC until use. Stock solution of lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4 (Sigma) was made up at 10 mg/ml in phenol-red-free Dulbecco’s Eagle medium (DMEM; Sigma) and stored at -20ºC until use. Stock solution of thioglycollate broth (LPS) from *Escherichia coli* serotype 0111:B4 (Sigma) was made up at 10 mg/ml in phenol-red-free Dulbecco’s Eagle medium (DMEM; Sigma) and stored at -20ºC until use. Thioglycollate broth (Merck, Germany) was prepared to a concentration of 3% (w/v) in L-15 medium, autoclaved at 121ºC for 10 min, and stored at room temperature until use. The arginine analogue N-monomethyl-L-arginine monoaetate (L-NMMA; Calbiochem, USA) was made up at 100 mM in DMEM, and stored until use at -20ºC in the dark. L-Glutamine, sulfanilamide and naphthylenediamine hydrochloride were likewise purchased from Sigma.

2.4. Elicitation and isolation of inflammatory peritoneal leucocytes

Inflammatory cells were elicited by i.p. injection of fish with 1 ml of 3% Brewer thioglycollate medium in incomplete L-15 medium, in some cases also containing 500 µg of protein per ml of TCL or ESP. Three days later, the fish were anaesthetized as indicated above and the anaesthesia was prolonged until death. Peritoneal leucocytes were then collected as described previously (Leiro *et al*., 2001), and the peritoneal fluid was maintained on ice. Cell concentration was counted using a haemacytometer and adjusted in Hanks’ balanced salts solution (HBSS) to 106 cells/ml by centrifugation at 500 x g for 15 min at 4ºC. Aliquots (100 µl) of the cell suspension were added to the wells of 96-well microculture plates (Corning, USA) and left for 60 min at 23ºC to allow adhesion. Non-adherent cells were then removed by two washes with 200 µl per well of HBSS, and more than 97% of the remaining adherent cells were viable (as determined using the trypan blue exclusion test); most of these cells are neutrophils and macrophages (Leiro *et al*., 2001).

2.5. Chemotaxis assay

Chemotaxis of turbot phagocytic cells was studied using a capillary-type microplate multiassay performed as previously described (Paramá *et al*., 2004). The assay was performed in sterile 96-well flat-bottomed plates (Sterilin, England), using a capillary tube with inverted U shape to connect two wells (A and B) containing A) 200 µl of peritoneal leucocytes (3 x 106 cell/ml) in L-15 medium and B) 200 µl of a solution of chemoattractant in L-15 medium. Migration of cells to the chemoattractant was assessed after 4 h incubation at 23ºC: leucocytes present inside the tubes and in the B wells were fixed with 1% glutaraldehyde, and the number of cells was determined using a Neubauer haemacytometer. Migration was expressed as chemotaxis index, i.e. number of migrated cells/random migration (random migration is defined as the number of cells that migrated towards the assay medium without chemoattractant in parallel control assays). All assays were performed in quintuplicate.

2.6. Peroxidase activity

Leucocytes present inside chemotaxis assay tubes were fixed for 1 min in PBS containing 1% formaldehyde and 2% glutaraldehyde, and peroxidase activity was determined as previously described (Leiro *et al*., 2000a). Briefly, fixed cells were incubated for 15 min in 0.15 M Tris buffer pH 7.6 containing 0.02% 3,3’-diaminobenzidine tetrahydrochloride and 0.006% H2O2. The slides were then washed three times with Tris buffer and contrasted with haematoxylin for 4 min. After dehydration with ethanol, permanent mounts were prepared with Eukitt. Stained cells (dark brown cytoplasm) were counted as peroxidase-positive.

2.7. Phagocytosis assay

Phagocytic activity was assayed by a previously described fluorometric method (Leiro *et al*., 2000a) using *K. lactis* yeast coupled to fluorescein isothiocyanate (FITC; Sigma). To couple FITC, yeast cells were suspended at 106 cells ml-1 in 50 mM sodium carbonate-bicarbonate buffer (pH 9.5) containing 150 mM NaCl and 40 mg of FITC. After incubation for 1 h at room temperature, the yeast suspension was centrifuged at 10000 x g for 5 min several times, each time discarding the supernatant and resuspending the pellet in PBS, until fluorescence in the supernatant dropped to zero. For assay, 100 µl of HBSS containing 106 FITC-labelled yeast cells was added to each well of 96-well microculture plates to which turbot peritoneal leucocytes (105 per well) had been coupled as described above, and incubated under optimal conditions for phagocytosis (optimal yeast-cell-to-phagocyte ratio, temperature, incubation time; Leiro *et al*., 1995). The wells were then washed several times with HBSS, and the cells were solubilized by adding 100 µl of 25 mM Tris-HCl pH 8.5 containing 0.2% sodium dodecyl sulphate (SDS). Fluorescence was measured in a microplate fluorescence reader (Bio-Tek Instruments; excitation 490 nm, emission 525 nm).
2.10. RNA isolation and cDNA production

One-thousand-microliter aliquots of 10^6 peritoneal inflammatory cells stimulated in vivo by injection of 3% Brewer thioglycollate medium in L-15 medium or containing 500 μg/ml of TCL or ESP, were incubated in microplates (Corning) at 23ºC under 5% CO₂ for 48 h in phenol-red-free DMEM containing 2 mM L-glutamine and 100 ng/ml of LPS or 500 μg/ml of ESP. In some wells, the arginine analogue L-NMMA (250 μM) was included in the medium as a reference inhibitor of nitric oxides (NO) production.

NO⁻ production in the culture supernatants was assayed by the Griess reaction (Green et al., 1982), by measurement of the total amount of inorganic NO⁻ as previously described (Leiro et al., 2004). Aliquots (100-μl) were removed from the medium and incubated with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine hydrochloride in 2.5% H₃PO₄) for 10 min at room temperature, and absorbance was then measured at 530 nm in an ELISA reader (Titertek Multiscan, Flow Laboratories, Finland). Nitrite concentration was calculated with reference to a standard curve obtained using NaNO₂ (1-200 μM of each primer and 1.5 U of rTaq DNA polymerase for 5 min, then centrifuged at 12000 x g for 15 min at 4ºC. The colourless aqueous upper phase was removed and RNA precipitated with isopropanol; the resulting RNA pellet was dried and dissolved in diethylpyrocarbonate (DEPC)-treated RNase-free water at a concentration of 1 μg/ml. cDNA synthesis (25 μl/reaction) was achieved using 1.25 μM random hexamer primers (Roche), 250 μM of each deoxyribonucleotide triphosphate (dNTPs), 10 mM DTT, 20 U of RNase inhibitor, 2.5 mM MgCl₂, 200 U of MMLV (murine leukaemia virus) reverse transcriptase (Promega) in 30 mM Tris and 20 mM KCl, pH 8.3, and 2 μg of sample RNA. The cycling parameters for the RT step were: hybridization for 10 min at 25ºC and reverse transcription for 60 min at 42ºC.

2.11. PCR amplifications, cloning and sequencing of turbot TGF-β

Initially, TGF-β1 of turbot was amplified by PCR using an arbitrary primers set designed for amplification of murine inflammatory cytokines (Rat Set 1, Biosource Europe, Belgium) as previously described (Leiro et al., 2003). The PCR products obtained were purified and cloned in the pGEM®-T easy system (Promega, USA), transformed in JM199 cells and sequenced as previously described (Leiro et al., 2002). Nucleotide and protein sequences are deposited in GenBank (National Center for Biotechnology Information, Bethesda, Maryland, USA) with accession number AF521669).

2.12. Expression analysis of pro-inflammatory cytokines in turbot inflammatory peritoneal leucocytes

For determination of cytokine gene expression we used a semi-quantitative RT-PCR (reserve transcription polymerase chain reaction). One μl of RT reaction mixture was amplified by PCR using the following forward/reverse primer pair: 5'-AACGGCCTCTACTCCGCTCTA-3' / 5'-GGCAGGTAGTGGGATCTTGTA-3' (TNF-α; GenBank of the National Center for Biotechnology Information, NCBI, accession number AB040449) (Hirono et al., 2000); 5'-ACAATCTCTGCGTATCCCTT-3' / 5'-CTCGTTCATGTCACTGGATGT-5' (TGF-β1; NCBI, accession number AF521669); 5'-TACCTGTCCTGCAACAGGAA-3' / 5'-TGATGAAACCAGGTTGGGAA-3' (IL-1β; NCBI, accession number AJ295836) and 5'-AACCTGGAATCGGACATGGAGA-3' / 5'-CTCAGGATCTCAGCAGGTTT-3' (β-actin, NCBI, accession number AJ295836)

2.8. Assays of ROS production

Extracellular ROS release by turbot peritoneal leucocytes was quantified using the fluorogenic reagent OxyBURST® Green H₂HFF BSA (Molecular Probes, The Netherlands) as previously described (Leiro et al., 2001). Peritoneal leucocytes (100 μl, 10⁶ cells/ml) were incubated with 10 μg/ml of OxyBURST® Green H₂HFF BSA for 2 min at 23ºC, then stimulated by addition of 10 μg/ml of PMA or, in some assays, 500 μg/ml of excretion-secretion products (ESP). Fluorescence emission was then determined in a microplate fluorescence reader (BioTek Instruments; excitation 488 nm, detection 530 nm, 55 min). The results are expressed as increase in fluorescence (arbitrary units) per min.

2.9. Assay of nitric oxides production

One-hundred microliter aliquots of peritoneal leucocytes that had been prestimulated in vivo by injection of 3% Brewer thioglycollate medium in L-15 medium or containing 500 μg/ml of TCL or ESP, were incubated in microplates (Corning) at 23ºC under 5% CO₂ for 48 h in phenol-red-free DMEM containing 2 mM L-glutamine and 100 ng/ml of LPS or 500 μg/ml of ESP. In some wells, the arginine analogue L-NMMA (250 μM) was included in the medium as a reference inhibitor of nitric oxides (NO) production.

NO⁻ production in the culture supernatants was assayed by the Griess reaction (Green et al., 1982), by measurement of the total amount of inorganic NO⁻ as previously described (Leiro et al., 2004). Aliquots (100-μl) were removed from the medium and incubated with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine hydrochloride in 2.5% H₃PO₄) for 10 min at room temperature, and absorbance was then measured at 530 nm in an ELISA reader (Titertek Multiscan, Flow Laboratories, Finland). Nitrite concentration was calculated with reference to a standard curve obtained using NaNO₂ (1-200 μM in culture medium).

2.10. RNA isolation and cDNA production

One-thousand-microliter aliquots of 10⁶ peritoneal inflammatory cells stimulated in vivo by thioglycollate or with TCL or ESP, were used for isolation of RNA. Isolation of total RNA from leucocyte samples was done with a monophasic solution of phenol and guanidine thiocyanate (TriPure Isolation Reagent, Roche). Briefly, 1 ml of TriPure was added to each well (area 10 cm²), and the cells were lysed by passing the suspension through a pipette several times. The lysate was incubated for 5 min at room temperature to ensure complete dissociation of nucleoprotein complexes. Subsequently 0.2 ml of chloroform was added for each 1 ml of TriPure, and the tube was vortexed vigorously for 15 sec, incubated at RT
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Thermal cycling in an automatic thermal cycler (GeneAmp PCR System 2400, Perkin-Elmer, Norwalk, USA) was as follows: initial denaturing at 94°C for 5 min; then 35 cycles at 94°C for 30 sec, 52°C (TNF-α, TGF-β and β-actin) or 45°C (IL-1β) for 45 sec, and 72°C for 1 min; and finally a 7-min extension phase at 72°C. In all experiments we performed controls without RNA or without reverse transcriptase; in no case were amplification products obtained. PCR products (20 µl aliquots) were separated on a 2% agarose gel in TBE buffer stained with 0.5 µg/ml of ethidium bromide, and photographed with a digital camera under a Spectroline 312 variable-intensity UV transilluminator (Spectroline) as previously described (Leiro et al., 2000b). mRNA amounts in each band were quantified using densitometry analysis software (ImageMaster Total Lab, ver. 2.00; AmershamPharmaciaBiotech), and expressed with respect to mRNA amount in the band corresponding to the control gene β-actin, run in the same gel.

2.13. Statistics

Data are expressed as means ± SEM. Means were compared (p = 0.05) by one-way ANOVA followed by Tukey-Kramer tests for multiple comparisons.

3. Results

3.1. Leucocyte chemotaxis in response to P. dicentrarchi extracts

We first performed in vitro assays to evaluate whether the P. dicentrarchi TCL and ESP preparations are chemoattractants for thioglycollate-induced turbot peritoneal leucocytes. To this end, groups of 5 turbot were intraperitoneally injected with 1 ml of 3% thioglycollate. On day 3 post-inoculation, peritoneal leucocytes were obtained, and we then assayed their chemotaxis towards TCL (protein concentration 500 µg/ml), ESP (protein concentration 500 µg/ml), or 10% normal turbot serum (NTS) in L-15 medium. The results obtained (Fig. 1A) indicate that both NTS and ESP have significant chemoattractant activity for turbot adherent leucocytes. By contrast, migration towards TCL was significantly lower than towards the control (L-15 medium alone) (Fig. 1A). About 43% of cells that migrated towards NTS showed peroxidase activity, versus only about 30% and 20% of cells that migrated towards ESP and TCL respectively (Fig. 1B).

In a second experiment we inoculated turbot with 3% thioglycollate plus L-15 medium, ESP (500 µg/ml), or TCL (500 µg/ml). On day 3 post-inoculation, peritoneal leucocytes were obtained for assay of chemotaxis towards L-15 medium, ESP (500 µg/ml), or TCL (500 µg/ml) respectively. The results obtained (Fig.2) indicate that chemotaxis was markedly and significantly higher for the ESP cells (prestimulated in vitro with ESP, presented with ESP in vitro) than for the TCL cells (prestimulated in vivo with TCL, presented with TCL in vitro). The specificity of the chemotaxis towards ESP is confirmed by the marked and significant difference between migration of ESP-prestimulated cells towards ESP and towards L-15 medium alone (Fig. 2).

3.2. Phagocytic activity of inflammatory leucocytes from turbot prestimulated with P. dicentrarchi extracts

In this experiment, turbot were inoculated in vivo with 3% thioglycollate containing a) L-15 medium alone, b) L-15 medium containing ESP (500 µg/ml), or c) L-15 medium containing TCL (500 µg/ml). On day 3 post-inoculation, peritoneal leucocytes were obtained
and phagocytic activity assayed by incubation with FITC-labelled *K. lactis* cells. The results of these assays (Fig. 3) indicate that leucocytes prestimulated with thioglycollate plus TCL showed significantly greater phagocytic activity than leucocytes prestimulated with thioglycollate plus ESP.

**3.3. ROS production by inflammatory leucocytes in response to stimulation in vivo and in vitro with *P. dicentrarchi* extracts**

Groups of 5 turbot were inoculated with 3% thioglycollate containing L-15 medium alone, L-15 medium plus ESP (500 µg/ml), or L-15 medium plus TCL (500 µg/ml). On day 3 post-inoculation, peritoneal leucocytes were obtained and stimulated *in vitro* with PMA (10 µg/ml) or ESP (500 µg/ml), with determination of extracellular ROS production using the fluorogenic reagent OxyBURST® Green H₂HFF BSA. In assays in which *in vitro* stimulation was with PMA, leucocytes prestimulated *in vivo* with thioglycollate plus TCL showed significantly higher ROS production than leucocytes prestimulated *in vivo* with thioglycollate plus ESP (Fig. 4, groups B and C). In assays in which *in vitro* stimulation was with ESP, only leucocytes prestimulated *in vivo* with thioglycollate plus TCL showed significantly raised ROS production with respect to the control (Fig. 4, group C).

**3.4. NO production by inflammatory leucocytes in response to stimulation in vivo and in vitro with *P. dicentrarchi* extracts**

For assays of NO production, cells were prestimulated in vivo by the same procedure as for the assays of ROS production. The cells were then incubated *in vitro* with LPS or with ESP, with determination of NO production by the Griess reaction. As shown in Fig. 5, NO production was highest by leucocytes prestimulated *in vivo* with thioglycollate plus ESP, regardless of
in vitro stimulation (Fig. 5, Group B). Leucocytes prestimulated in vivo with TCL showed markedly lower NO production than leucocytes prestimulated in vivo with ESP, and indeed than leucocytes prestimulated in vivo with thioglycollate only (Fig. 5, Group A versus Groups B and C).

Independently of the in vivo prestimulation, NO production was highest when there was no in vitro stimulation; in Group B, for example, NO production in the presence of LPS or ESP was significantly lower than in the presence of L-15 medium only (Fig. 5, Group B, bars 6 and 7 versus bar 5).

**3.5. Time-course of inflammatory cytokine mRNA levels in inflammatory leucocytes**

To investigate the time-course of expression of the pro-inflammatory cytokines TNF-α and IL-1β, and of the anti-inflammatory cytokine TGF-β, in thioglycollate-induced leucocytes, we monitored levels of the mRNAs of these cytokines by semiquantitative RT-PCR. The results shown in Fig. 6 indicate that mRNA levels of the pro-inflammatory cytokines peaked around day 3 post-inoculation with thioglycollate, while mRNA levels of TGF-β peaked around day 1.

**3.6. Inflammatory cytokine mRNA levels after in vivo stimulation with *P. dicentrarchi* extracts**

For this part of the study we determined mRNA levels of the cytokines TNF-α, IL-1β and TGF-β in leucocytes from turbot inoculated with L-15 medium only, L-15 medium containing ESP (500 µg/ml), or L-15 medium containing TCL (500 µg/ml). Leucocytes were obtained 3 days post-inoculation, and mRNA levels estimated by semi-quantitative RT-PCR. As shown in Fig. 7, mRNA levels of all three cytokines were significantly higher in leucocytes from turbot stimulated in vivo with thioglycollate plus ESP than in leucocytes from the control turbot (stimulated in vivo with thioglycollate only; Fig. 7, Group B). In the case of leucocytes from turbot stimulated in vivo with thioglycollate plus TCL, a) mRNA levels of the anti-inflammatory cytokine TGF-β were significantly higher than both the control turbot and the ESP-stimulated turbot (Fig. 7, Group C, bar 7); b) mRNA levels of the pro-inflammatory cytokine IL-1β were significantly lower than both the control turbot and the ESP-stimulated turbot (Fig. 7, Group C, bar 8); and c) mRNA levels of the pro-inflammatory cytokine TNF-α were significantly lower than in the ESP-stimulated turbot but did not differ significantly from the control turbot (Fig. 7, Group C, bar 9).
peritoneal leucocytes. Several previous studies have found that parasite extracts induce polarization of fish leucocytes, suggesting the presence of chemoattractants (Taylor & Hoole, 1993). The principal components of parasite excretion-secretion products typically include proteases, which in *P. dicentrarchi* show marked cysteine proteinase activity (Paramá et al., 2004). However, the few studies that have been performed to date with proteases secreted by different parasites in human peripheral blood monocytes and neutrophils have indicated that these proteases are involved in inhibition of chemotactic responses and the oxidative burst response (Shepard et al., 1991; Sorensen et al., 1994). The inflamed peritoneal exudates of fish were mainly composed of leucocytes, of which about 32% were neutrophils, about 26% macrophages and about 23% lymphocytes, with only the neutrophils being peroxidase-positive (Vazzana et al., 2003). Intraperitoneal injection of fish with inflammatory substances augments the proportion of neutrophils (Afonso et al., 1998; Peddie et al., 2002). In the present study we observed that turbot neutrophils showed strong migratory activity in the absence of serum or other chemoattractants, in line with Hamdani et al. (1998), while prestimulation *in vivo* with the inflammatory agent thioglycollate increased the proportion of neutrophils showing chemotaxis. However, the proportion of neutrophils among the migratory cells declined significantly when parasite extracts (ESP or TCL) were offered as candidate chemoattractants. Among the different types of leucocyte present in fish peritoneal exudate (macrophages, neutrophils, eosinophilic granular cells, lymphocytes, and thrombocytes), only macrophages and neutrophils show significant phagocytic capacity (Do Vale et al., 2002). Although the viable forms of some parasites suppress fish phagocyte function (Leiro et al., 2001; Fast et al., 2002; Gross et al., 2004), extracts of these same parasites may stimulate phagocyte function (Scharssack et al., 2003). In the present study, only peritoneal leucocytes from turbot prestimulated *in vivo* with the whole parasite lysate (TCL) showed significantly increased phagocytic activity, while peritoneal leucocytes from turbot prestimulated with excretion-secretion products (ESP) did not show any increase in phagocytic activity.

Depending on the cytokine environment, phagocytic cells (especially macrophages) can differentiate into distinct subsets, which can be induced by pro-inflammatory microbial molecules (e.g. LPS) or cytokines (e.g. IFN-γ or TNF-α) to release inflammatory and/or microbicidal products, such as ROS (notably the superoxide anion $O_2^-$), reactive nitrogen species (notably nitric oxide) and pro-inflammatory cytokines (e.g. TNF, IL-1 and IL-6). The persistent presence of these products may result in tissue damage (Noël et al., 2004). Although still rather poorly understood, the respiratory burst of fish leucocytes

![Figure 7](image-url) **Fig. 7.** mRNA levels of interleukin 1 beta (IL-1β), tumour necrosis factor alpha (TNF-α) and transforming growth factor beta (TGF-β) in peritoneal leucocytes from turbot inoculated 3 days previously with 3% thioglycollate plus A) L-15 medium only, B) *P. dicentrarchi* excretion-secretion products (ESP), or C) *P. dicentrarchi* whole extract (TCL). Cytokine levels were determined by RT-PCR. A) Agarose gel (2%) analysis of RT-PCR products using primers for IL-1β (181 bp), TNF-α (100 bp), TGF-β (243 bp) or the housekeeping gene β-actin (330 bp). B) mRNA levels as determined by densitometric analysis [optical density (OD) of cytokine band, divided by OD of band obtained in parallel assay using primer for β-actin]. Values shown are means ± standard errors (n = 5 assays); asterisks indicate significant differences (*, *p* < 0.05; **, *p* < 0.01) with respect to the groups indicated in brackets; numbers at the base of each bar are group numbers.3.
activates NO\textsuperscript{2} (Leiro et al., 2004). Likewise, NO\textsuperscript{2} stimulates NO\textsuperscript{•} production in vivo with P. dicentrarchi excetration-secretion products (ESP), and inhibited in leucocytes prestimulated with the whole parasite lysate (TCL). This apparent difference in responses to the two parasite extracts may be due to the fact that the Griess reaction measures levels of NO\textsubscript{2} and NO\textsubscript{3} after breakdown of NO\textsuperscript{•} in aqueous solution (James, 1995); however, if there is a high concentration of the superoxide anion O\textsubscript{2}\textsuperscript{-}, as occurs in cells prestimulated with TCL, the O\textsubscript{2}\textsuperscript{-} may react with NO\textsuperscript{•} to form peroxynitrite (ONOO\textsuperscript{-}), which is not detected in the Griess reaction; in other words, the apparently low NO\textsuperscript{•} production in TCL-prestimulated cells may be a false negative result.

Previous histological studies have demonstrated that infections of turbot with P. dicentrarchi are followed by marked inflammation of the brain tissues. Inflammation of brain tissues is also common in fish infected with other ciliates, such as Ichthyophthirius multifiliis (Sigh et al., 2004). Inflammation is a complex defence mechanism involved in the destruction of pathogens and other foreign bodies: acute inflammation is a generally beneficial response, but chronic inflammation may progress to inflammatory disease (Kaplanski et al. 2003). Cytokines are a functionally diverse group of cellular messengers that act through the janus kinase signal transducer and activator of transcription (JAK/STAT) and are integral components of adaptive and innate immune responses (Alexander & Hilton, 2004). ROS and pro-inflammatory cytokines act synergistically and contribute to the onset and progression of inflammation in various organs (Closa & Folch-Puy, 2004). In mammals, it has become evident that interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-\(\alpha\)), mainly produced by monocytes/macrophages, are the principal mediators of tissue destruction in many immune-inflammatory diseases (Dayer & Burger, 1994). In parasite infections by monogeneans it has been proposed that the release of cytokines (e.g. IL-1, TNF and IFN) initiates a series of events leading to a decrease in the ectoparasite population (Buchmann, 1999), and the pro-inflammatory cytokine IL-1\(\beta\) has been postulated as an immune adjuvant (Ying & Kwang, 2000). In rainbow trout, Oncorhynchus mykiss, the pro-inflammatory cytokine IL-1\(\beta\) is specifically expressed during infection with the monogenean Gyrodactylus derjavini (Lindenstrom et al., 2003). In this fish species, release of TNF has also been seen during primary infections with G. derjavini (Lindenstrom et al., 2004), and during a natural outbreak of proliferative kidney disease (PD) due to a myxozoan parasite (Holland et al., 2003). Carp intraperitoneally injected with the flagellate parasite Trypanoplasma borelli up-regulate expression of the TNF-\(\alpha\) and IL-1\(\beta\) genes (Saeij et al., 2003a); however, during infection with the bacteria Renibacterium salmonarum, macrophages...
showed a rapid inflammatory response in which the expression of IL-1β was enhanced but TNF-α expression was greatly reduced (Grayson et al., 2002), indicating a possible mechanism for modulation of the host immune response by the parasite. Transforming growth factor beta (TGF-β) is an immunoregulatory cytokine with primarily suppressive properties, including down-regulation of MHC expression, production of some cytokines, T and B cell proliferation, IgG and IgM production, IL-2 receptor expression, cytotoxic T-cell generation and function, LK and NK cell activation and function, macrophage activation, and macrophage respiratory burst activity (Ruscetti & Palladino, 1991). However, TGF-β has some pro-inflammatory effects, including promotion of macrophage and neutrophil chemotaxis, IgA production, and production of some cytokines (Harms et al., 2003). Whether TGF-β has pro- or anti-inflammatory effects depends on its concentration, the differentiation status of the target cells, and the concentration of other pro-inflammatory compounds; In any case, it plays a critical immunoregulatory role by limiting damage to tissues during resolution of the normal immune response (McCartney-Francis & Wahl, 1994). TGF-β isoforms (TGF-β1, β2 and β3) have been described in many fish species (Tafalla et al., 2003). The present study clearly shows that transcription of the IL-1β, TNF-α and TGF-β genes occurs in thioglycollate-induced turbot peritoneal leucocytes, peak levels of IL-1β mRNA and TNF-α mRNA appearing around 3 days after inoculation with thioglycollate, or around 1 day post-inoculation in the case of TGF-β. Transcription of the IL-1β gene began within 24 hours; however, transcription of the TNF-α gene was not detected during this period. In the sea bass Dicentrarchus labrax, transcription of the IL-1β gene was likewise detected within 24 hours of in vivo stimulation with LPS (Scapigliati et al., 2001). In the present study, mRNA levels of all three cytokines declined over time, in each case dropping significantly below the peak level by day 7 post-inoculation. When turbot were inoculated in vivo with thioglycollate plus P. dicentrarchi excretion-secretion products (ESP), mRNA levels of all three cytokines reached significantly higher levels than in control turbot inoculated with thioglycollate only. When turbot were inoculated in vivo with thioglycollate plus the P. dicentrarchi whole extract (TCL), mRNA levels of IL-1β and TNF-α were close to the levels seen in control turbot, while mRNA levels of TGF-β were significantly higher than in the ESP-stimulated turbot. Increased expression of the TNF-α gene has similarly been observed in a previous study of rainbow trout infected with the monogenean Gyrodactylus derjavini (Lindenstrom et al., 2004). Likewise, intraperitoneal inoculation of carp with lysates of Trypanoplasma borelli induces expression of IL-1β and TNF-α in phagocytes (Saeij et al., 2003a), while rainbow trout infected with the ciliate Ichthyophthirius multifiliis showed increased IL-1β and TNF-α expression by day 4 after infection (Sigh et al., 2004).

In conclusion, the results of the present study suggest that the immunopathogenic effects of Philasterides dicentrarchi infection are attributable to the parasite’s excretion-secretion products. These effects may reflect over-expression of proinflammatory cytokines and over-production of NO and O₂ phagocytic cells. Under this hypothesis, as parasites are destroyed by the immune system, there will be an increase in the endocytic activity of phagocytic cells, and up-regulation of the expression of TGF-β, which has been shown to play a role in abating iNOS and thus inhibition of NO production (Leiro et al., 2003); these processes would thus tend to block the acute inflammatory response to the infection.

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6. References


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