Acidophilic granulocytes of the marine fish gilthead seabream (*Sparus aurata* L.) produce interleukin–1β following infection with *Vibrio anguillarum*

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Abstract

The fish immune response to Gram-negative bacteria is poorly understood. In this study, we use a monoclonal antibody (mAb) specific to acidophilic granulocytes from the marine fish gilthead seabream (*Sparus aurata* L.), together with an antiserum specific to interleukin-1β (IL-1β) from this species, in order to investigate whether these cells are involved in the immune response against the pathogenic bacterium *Vibrio anguillarum* and, in particular, in the production of the pro-inflammatory cytokine IL-1β. We found that gilthead seabream head-kidney, peritoneal exudate and peripheral blood leukocytes accumulated proIL-1β intracellularly when challenged in vitro with *V. anguillarum*, whereas only peritoneal exudate and blood leukocytes were able to accumulate proIL-1β following infection. Importantly, the blood leukocytes from infected animals that accumulated proIL-1β were shown to be the acidophilic granulocytes. A rapid mobilization of such cells from the head-kidney to the site of inflammation following infection with *V. anguillarum* was also observed.

Keywords: Acidophilic granulocytes, IL-1β, *Vibrio anguillarum*, Fish, *Sparus aurata* L. (Teleostei)

Introduction

Vibriosis caused by *Vibrio anguillarum* is one of the major diseases in aquaculture, where it affects not only fish but also bivalve molluscs and crustaceans (Toranzo and Barja 1990). Although several commercial vaccines against *V. anguillarum* are widely used and provide some protection against infection, little is know about the mechanisms underlying this immune protection. Previous studies have demonstrated that antibodies contribute to protection against *V. anguillarum* serogroups 01 (Akhlaghi 1999) and 02 (Boesen et al. 2001) in rainbow trout passively immunised with *V. anguillarum* antiserum. Activation of the complement system by the classical pathway has been suggested as the protection mechanism induced by vaccination against *V. anguillarum* serogroup 01, since sub-agglutinating antibody levels in the presence of complement have been shown to kill this serogroup in vitro (Boesen et al. 1999a). However, this seems to be unlikely in the case of *V. anguillarum* serogroup 02, since this serogroup is resistant to in vitro serum killing even in the presence of antibodies (Boesen et al. 1999b) and neither antibodies nor complement are able to play an opsonising effect (Boesen et al. 2001). The authors of these studies suggest that antibodies may prevent the attachment of the pathogen to the host's tissues and/or that neutrophils may play a role either by the direct phagocytosis of bacteria or by transferring toxic components to the macrophages (Afonso et al. 1998). Therefore, more studies are needed to understand fish innate and adaptive immune responses against this pathogenic bacterium.

To date, the role played by fish granulocytes against bacterial infections has not been properly established because of the marked morphological heterogeneity of these cells between different species, and the lack of specific cell surface markers. It had previously been reported using electron microscopy and flow cytometry studies, that gilthead seabream (*Sparus aurata* L.) acidophililic granulocytes are able to engulf *V. anguillarum* R82 serogroup 01 (Esteban et al. 1998), which, together with the morphological and cytochemical features of these cells (López-Ruiz et al. 1992; Meseguer et al. 1994), suggests that they may be functionally equivalent to the neutrophils of higher vertebrates. This seems to be further confirmed thanks to the availability of a monoclonal antibody (mAb) specific to these cells, which has made it

possible to demonstrate that acidophilic granulocytes constitute 85% of gilthead seabream head-kidney leukocytes showing in vitro phagocytic activity towards *V. anguillarum* R82 (Sepulcre et al. 2002). Therefore, a prominent role for the acidophilic granulocytes of the gilthead seabream during *V. anguillarum* infection is to be anticipated according to the above in vitro results.

In this study, we report that gilthead seabream peripheral blood and peritoneal exudate acidophilic granulocytes accumulated proIL-1β intracellularly in response to *V. anguillarum* infection, whereas head-kidney leukocytes did not, in spite of showing increased IL-1β transcript accumulation. In addition, acidophilic granulocytes were rapidly mobilized from the head-kidney to the site of inflammation, following infection with *V. anguillarum*.

Materials and methods

Animals

Healthy specimens (100 g mean weight) of the hermaphroditic protandrous marine teleost gilthead seabream (*Sparus aurata* L.) obtained from Culmarex (Murcia, Spain) were kept in 260-l running seawater aquaria (flow rate 1,500 l/h) at 20^{o}C with a 12-h light/dark cycle. Fish were fed with a commercial pellet diet (Trouvit, Spain) at a feeding rate of 15 g dry diet/kg biomass of fish/day.

Cell culture and treatments

Head-kidney and peritoneal exudate leukocytes obtained as described elsewhere (Sepulcre et al. 2002) were stimulated for 0.5, 1, 2, 4, 8 and 16 h at 25^{o}C with formalin-killed *V. anguillarum* R82 (serogroup 01) (Toranzo and Barja 1990) cells at a ratio of 1:1 or 1:10 (leukocyte:bacteria) in sRPMI [RPMI-1640 culture medium (Gibco) adjusted to gilthead seabream serum osmolarity (353.33 mOs) with 0.35% NaCl] supplemented with 5% fetal calf serum (FCS, Gibco), and 100 IU/ml penicillin and 100 μg/ml streptomycin (Biochrom). At the end of the incubation, the cells were extensively washed in phosphate-buffered saline (PBS) and lysed either with cold TRIzol Reagent (Invitrogen) for PCR analysis or boiled in SDS-sample loading buffer for immunoblot analysis. Supernatants were also collected, passed through a 0.22 μm filter, concentrated by precipitation with 20% trichloroacetic acid (Sigma) and boiled for 5 min in SDS-sample loading buffer for immunoblot analysis.

Experimental infections

Fish were injected intraperitoneally (i.p.) with 10^8 exponentially growing *V. anguillarum* R82 cells. Head-kidney, blood and peritoneal exudate cells, and supernatants from individual peritoneal cavity lavages were obtained 16 h after bacterial challenge unless otherwise indicated. Peripheral blood leukocytes were isolated by using 50% Percoll density gradients (Pharmacia). Cell suspensions were processed as described above for subsequent RT-PCR,

immunoblot and/or flow cytometry analysis. Fragments of head-kidneys and blood smears were also obtained and processed for the immunohistochemistry study (see below).

RT-PCR

The SuperScript II RNase H^- reverse transcriptase (Invitrogen) was used to synthesise first strand cDNA with an oligo-dT17 primer from 1 μg of total RNA at 42°C for 50 min. The cDNA preparations were used in PCR amplification with primers specific for gilthead seabream IL−1β (5'-ATGCCCGAGGGGCTGGGC-3' and 5'-CAGTTGCTGAAGGGAACAGAC-3') and β-actin (5'-ATCGTGGGGCGCCCCAGGCACC-3' and 5'-CTCCTTAATGTCACGCACGATTTC-3') genes (Pelegrín et al. 2001). Amplification was performed in 50-μl samples containing 5 μl of PCR reaction buffer (10x, Ecogen), 2 μl forward and reverse primers (10 μM each), 1 μl dNTP mix (2.5 mM each), 2.5 μl $MgCl_2$ (50 mM), 0.5 μl TaqDNA polymerase (5 U/μl, Ecogen), 34 μl DNase/RNase-free distilled water (Sigma) and 3 μl cDNA template (corresponding to 10 ng of RNA). The cycling reaction was performed in a MasterCycler Gradient (Eppendorf) for one cycle of 94°C for 2 min, 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by one cycle of 72°C for 10 min. PCR products were separated in a 1% agarose gel containing 0.5 mg/ml ethidium bromide (Sigma) and visualised under UV light.

Western blot analysis

Cell extracts and concentrated supernatants (50 mg per lane) were run in 15% SDS-PAGE and transferred for 50 min at 200 mA to nitrocellulose membranes (BioRad). The blots were developed using a 1/1,000 dilution of a rabbit polyclonal antiserum to gilthead seabream IL−1β (anti-sbIL−1β) (Pelegrín et al. 2004), and enhanced chemiluminescence (ECL) reagents (Amersham Biosciences) according to the manufacturer's protocol. Membranes were then stained with a 0.1% Ponceau solution (Sigma) and/or re-probed with a 1:10,000 dilution of a monoclonal anti-β-actin (A5441, Sigma) to confirm a similar protein loading in all lanes.

Light microscopy and immunohistochemical staining

The head-kidney fragments were fixed in Bouin-Hollande fluid, embedded in Paraplast Plus (Sherwood Medical) and sectioned at 5 mm. After de-waxing and rehydration, the sections were subjected to an indirect immunocytochemical method (Sternberger 1986) by using a mAb specific to gilthead seabream acidophilic granulocytes (G7) (Sepulcre et al. 2002). Blood smears were fixed in methanol and stained with Giemsa (Merck) or subjected to the peroxidaseantiperoxidase (PAP) technique (Sternberger 1986) by using the anti-sbIL-1β (Pelegrín et al. 2004) or to a double immunofluorescence technique with these two primary antibodies.

The samples were incubated for 40 min in peroxidase quenching solution (H_2O_2 in methanol, 1:9) to eliminate the endogenous peroxidase and then rinsed in Coons buffer (0.01 M sodium diethylbarbiturate, 0.1 M NaCl, pH 7.4) and in Coons buffer containing 0.01% BSA and 0.2% Triton X-100 (CBT). After a 30-min incubation with skimmed milk powder in Coons buffer to block the non-specific reaction, they were rinsed in CBT and incubated for 2 h at room temperature with the first antiserum, at the optimal dilution of 1:10 (G7) or 1:100 (anti-sbIL-1β). After washing in CBT, the blood smears that had been incubated with anti-sbIL-1β were exposed to swine anti-rabbit IgG (Dako) diluted 1:20 with CBT for 1 h at room temperature, washed with CBT, and incubated with rabbit peroxidase-antiperoxidase complex (Dako) diluted 1:100 with CBT for 1 h at room temperature. The head-kidney sections that had been incubated with G7 were exposed to anti-mouse IgG (whole molecule) peroxidase conjugate (Sigma) diluted 1:100 for 1 h at room temperature. All the samples were then washed in CBT and in 0.5 M TRIS-HCl buffer (pH 7.6), and the peroxidase activity revealed by incubation with 0.05% 3,30-diaminobenzidine tetrahydrochloride (DAB) in TRIS-HCl buffer (pH 7.6) containing 0.05% H_2O_2 for 15 min at room temperature. The sections were slightly counterstained with haematoxylin. Samples were examined with an Axiolab microscope (Zeiss).

For the double immunofluorescence, blood smears were fixed in methanol and then incubated with anti-sbIL-1β and/or G7 antibodies, followed by fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG (Sigma) and/or tetramethylrhodamine isothiocyanate (TRICT)-labelled anti-rabbit Igs (Dako). Nuclei were then stained with 40,6-diamidino-2-phenylindole (DAPI, Sigma). The specificity of the reactions was determined by omitting the first antisera. Samples were examined with a DMRB fluorescence microscope (Leica).

Flow cytometry

Aliquots of 10^5 cells were washed in PBS containing 2% FCS and 0.05% sodium azide (FACS buffer), incubated for 20 min on ice with 100 ml of a 1/100 dilution of G7 mAb, washed and incubated with 50 μl of a 1/100 dilution of FITC-labelled anti-mouse IgG F(ab0)2 fragment of goat antibody (Sigma) for 20 min on ice. Cells were then washed twice and data collected in the form of two parameter forward-scatter (FSC) and side-scatter (SSC) dot plot and green fluorescence (FL1) or red fluorescence (FL2) histograms using a fluorescence-activated cell sorter (Becton Dickinson). The specificity of staining was checked by using a mAb isotype standard.

Protein determination

The protein concentrations of cell lysates and concentrated supernatants were estimated by the BCA protein assay reagent (Pierce) using BSA as a standard.

Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) and unpaired Student's t-test to determine differences between groups. A quantitative study of the flow cytometry results was made by using the statistical option of the Lysis Software Package (Becton Dickinson).

Results

sbIL−1β is intracellularly accumulated by *V. anguillarum*-challenged leukocytes

RT-PCR analysis was used to examine the kinetics of IL−1β transcript accumulation in head-kidney cells following *V. anguillarum* stimulation (Fig. 1a). Cells incubated for 4 h in the medium in the absence of *V. anguillarum* resulted in a weak accumulation of IL−1β transcript. Nevertheless, the amount of IL−1β transcript increased significantly upon the addition of formalin-killed *V. anguillarum*. This was further confirmed by immunoblot analysis, where incubation of gilthead seabream leukocytes from both head-kidney and peritoneal exudate with formalin-killed *V. anguillarum* for 16 h resulted in the intracellular accumulation of proIL−1β at both leukocyte:bacteria ratios used (Fig. 1b). The effect of *V. anguillarum* on proIL−1β accumulation by head-kidney cells was found to be time-dependent, with significantly greater increases of proIL−1β accumulation being observed after 8 h of stimulation (Fig. 1c). Notably, cells incubated for 16 h in medium alone did not accumulate detectable quantities of proIL−1β (Fig. 1b, c), despite expressing the IL−1β gene at low levels (Fig. 1a). Under these experimental conditions, IL−1β was never detected in the supernatants obtained from control or bacterially challenged leukocytes (data not shown).

The above results prompted us to examine whether IL−1β was also produced in vivo during the course of infection by *V. anguillarum*. The results show that IL−1β gene expression was up-regulated 16 h post-infection in both head-kidney and peritoneal exudate cells from infected fish (Fig. 2a), whereas they did not accumulate significant amounts of IL−1β polypeptide (Fig. 2b). Moreover, IL−1β was not detected in the supernatants from individual peritoneal cavity lavages (data not shown). In sharp contrast, *V. anguillarum* infection resulted in the intracellular accumulation of proIL−1β by peripheral blood leukocytes 16 h post-infection (Fig. 2b), as we have previously reported for the IL−1β transcript by using RT-PCR (Pelegrín et al. 2001). A more detailed kinetic study of IL−1β production shows that IL−1β was also intracellularly accumulated by peritoneal exudate cells as early as 4 h post-injection, although it returned to basal levels soon after (i.e. 16 h post-injection) (Fig. 2c). However, head-kidney

leukocytes from infected fish did not accumulate intracellular IL-1β at any time during the experiment (i.e. from 4 to 72 h) (data not shown).

Blood acidophilic granulocytes from *V. anguillarum*-infected fish produce sbIL-1β

Giemsa-stained blood smears from control and *V. anguillarum*-infected fish (Fig. 3a) revealed the presence of cells showing an eccentric nucleus and an acidophilic granulated cytoplasm. These morphological features are typical of the gilthead seabream acidophilic granulocytes (López-Ruiz et al. 1992) and this was further confirmed by immunohistochemistry, since these cells specifically reacted with the G7 (Fig. 4a), a mAb specific to the gilthead seabream acidophilic granulocytes (Sepulcre et al. 2002). Interestingly, circulating acidophilic granulocytes from infected fish reacted with the sbIL-1β antiserum (Fig. 3c), while blood acidophilic granulocytes from non-infected fish did not (Fig. 3b). Finally, the double immunofluorescence study confirmed that blood acidophilic granulocytes (G7+ cells) from infected fish reacted with the sbIL-1β antiserum (Fig. 4). In addition, all the above studies also showed that erythrocytes, lymphocytes, thrombocytes and monocyte/macrophages (G7-cells) from control and infected fish were unable to react with the sbIL-1β antiserum.

Acidophilic granulocytes are rapidly mobilized from the head-kidney after *V. anguillarum* infection

The above light-microscopy studies suggest an active involvement of gilthead seabream acidophilic granulocytes in the first stages of an infection with *V. anguillarum*. Therefore, it is tempting to hypothesise that these cells may be rapidly mobilized from the main granulopoietic organ in teleost fish, the head-kidney (Zapata et al. 1996), to the site of inflammation. This was evaluated by immunohistochemistry and flow cytometry using the G7 mAb. The results show that acidophilic granulocyte abundance and location were related to the infection (Fig. 5, 6). Thus, this cell type was seen to be abundant and occur in clusters in the head-kidney of non-infected fish (Fig. 5a), whereas they appeared isolated and scattered in the head-kidney

of infected fish (Fig. 5b). In fact, the percentage of acidophilic granulocytes decreased in the head-kidney and concomitantly increased in the peritoneal exudate 16 h after the infection (Fig. 6). Unexpectedly, the percentage of peripheral blood acidophilic granulocytes was similar in both control and infected fish (Fig. 6). The mean fluorescence intensity of cells stained with the G7 mAb was similar in cells from control and infected fish, ruling out the possibility that infection might affect the staining of acidophilic granulocytes, thus leading to misinterpretation of the percentages of these cells in the immune tissue of infected fish.

Discussion

The immune response of fish against *V. anguillarum* is largely unknown, despite the fact that it is one of the most menacing bacteria in aquaculture. Several studies have demonstrated that antibodies generated as part of the adaptive immune response contribute to the protection of fish against infection (Akhlaghi 1999; Boesen et al. 2001), but the mechanisms involved in this immune protection have proved to be elusive and, moreover, seem to depend on each particular *V. anguillarum* serogroup.

The results obtained in the present study suggest a role for the acidophilic granulocytes of the gilthead seabream during the first stages of infection with *V. anguillarum* serogroup 01, when antibodies are expected to be absent, at least in non-immunized fish. The rapid mobilization of acidophilic granulocytes following infection to the site of inflammation is not surprising, since previous studies have suggested that these cells may be functionally equivalent to the neutrophils of higher vertebrates (Esteban et al. 1998; López-Ruiz et al. 1992; Meseguer et al. 1994). Moreover, they are the most numerous phagocytic cell type in several immune organs of the gilthead seabream and, more importantly, they constitute 85% of headkidney leukocytes of this species able to phagocytose *V. anguillarum* R82 in vitro in the absence of both specific antibodies and complement (Sepulcre et al. 2002). Although the phagocytic activity of the two professional phagocytes of this species, i.e. monocyte/macrophages and acidophilic granulocytes, has not been determined to date, the rapid mobilization of acidophilic granulocytes to the site of inflammation, together with their known tissue abundance and in vitro phagocytic activity towards *V. anguillarum* (Sepulcre et al. 2002) in serum-free conditions, suggest an important role for these cells in the rapid clearance of this bacteria even in the absence of specific antibodies and complement activation.

IL-1β is a pro-inflammatory cytokine, whose functional role is largely unknown in fish. Recent studies have demonstrated that trout recombinant IL-1β increases the expression levels of IL-1β, cyclooxygenase 2 and MHC class II b-chain transcription in a rainbow trout macrophage cell line (RTS11), as well as the phagocytic activity of rainbow trout head-kidney leukocytes

(Hong et al. 2001). In this study, we show for the first time that IL-1β is intracellularly accumulated by peritoneal exudate and peripheral blood leukocytes following *V. anguillarum* infection. In contrast, head kidney leukocytes from infected fish do not accumulate IL-1β, despite showing increased IL-1β transcript accumulation. The intracellular accumulation of IL-1β by peritoneal exudate cells exclusively 4 h post-infection is particularly surprising taking into account that the mature cytokine is not detected in the supernatants from the peritoneal cavity lavages 16 h post-infection. However, the possibility cannot be ruled out that IL-1β is actually released by peritoneal exudate cells from infected animals, and is rapidly degraded or, alternatively, it is not accumulated in sufficiently great amounts to be detected.

The production and secretion of IL-1β have not been studied in fish due to the lack of specific antibodies. In mammals, the mechanism of release of this cytokine is quite controversial. Unlike most secreted proteins, it lacks a secretory signal sequence and does not follow the classical endoplasmic reticulum to Golgi route of secretion (Rubartelli et al. 1990). IL-1β is produced mainly by activated macrophages as a 31-kDa biologically inactive precursor molecule (proIL-1β) proteolytically cleaved to a 17-kDa active form by IL-1β-converting enzyme (ICE, caspase-1) (Cerretti et al. 1992). Although the levels of basal processing and release of IL-1β are quite low, they are dramatically induced by the presence of extracellular ATP, which can be autocrinally generated by activated monocytes, and which interacts with P2X7 purinergic receptors (Ferrari et al. 1997). Strikingly, all non-mammalian IL-1β genes cloned so far lack a conserved ICE recognition site (Bird et al. 2002). We have recently demonstrated that addition of extracellular ATP does not promote IL-1β secretion by gilthead seabream leukocytes, whereas a gilthead seabream SAF-1 fibroblast cell line shed microvesicles containing a 22-kDa IL-1β form within 30 min of activation with ATP (Pelegrín et al. 2004). In the present study, we show that peritoneal exudate cells and peripheral blood leukocytes from infected fish accumulate IL-1β intracellularly, and this suggests that a second stimulus is needed in vivo for IL-1β secretion in fish, as occurs in mammals.

Another important finding of this study is that the gilthead seabream peripheral blood leukocytes, which intracellularly accumulate IL−1β following infection with *V. anguillarum*, are acidophilic granulocytes. Although activated monocytes and macrophages are the main source of IL−1β in mammals (Dinarello 1996) and the IL−1β gene is highly expressed by activated gilthead seabream (Pelegrín et al. 2001) and rainbow trout (Zou et al. 1999) macrophages, both carp macrophages and neutrophilic granulocytes were shown to produce an IL-1-like molecule (Verburg-van Kemenade et al. 1995). Whatever the case, our data show that IL−1β is produced by peripheral blood acidophilic granulocytes following infection with *V. anguillarum*, but do not rule out the possibility that other cell types, such as monocytes/macrophages or even thrombocytes produce this cytokine at lower levels, undetectable by immunocytochemistry. Our data point to the need for further research to clarify our knowledge about the contribution of different immune cells and IL−1β in relation to protection of fish against *V. anguillarum* infection.

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Figure Legends

Figure 1. a–c ProIL−1β is intracellularly accumulated by leukocytes challenged in vitro with *V. anguillarum*. **a** RT-PCR analysis of IL−1β transcript accumulation by head-kidney cells challenged in vitro with formalin-killed *V. anguillarum* for the indicated times. **b** Western blot analysis of whole lysates obtained from $5x10^6$ head-kidney or $2.5x10^6$ peritoneal exudate cells incubated for 16 h in the absence or presence of formalin-killed *V. anguillarum* cells at the indicated leukocyte:bacteria ratio. **c** Western blot analysis of whole lysates obtained from $5x10^6$ head-kidney cells incubated in the presence of formalin-killed *V. anguillarum* cells at the indicated incubation times. The results are representative of three independent experiments.

Figure 2. **a–c** ProIL−1β is intracellularly accumulated by peripheral blood leukocytes following infection with *V. anguillarum*. **a** RT-PCR analysis of IL−1β transcript accumulation by head-kidney and peritoneal exudate leukocytes of control specimens or following 16 h challenge with *V. anguillarum*. NT no template. **b** Western blot analysis of whole lysates obtained from $5x10^6$ head-kidney cells, $2.5x10^6$ peritoneal exudate cells or $5x10^6$ blood leukocytes of one control and two infected specimens following 16 h challenge with *V. anguillarum*. **c** Western blot analysis of whole lysates obtained from $2.5x10^6$ peritoneal exudate leukocytes of control specimens or 4, 24, 48 or 72 h after challenge with *V. anguillarum*. The results are representative of three fish.

Figure 3. a–c Blood leukocytes showing an acidophilic granulated cytoplasm accumulate IL−1β intracellularly following infection with *V. anguillarum*. Blood smears from control specimens (**b**) or following 16-h challenge with *V. anguillarum* (**a, c**) were stained with Giemsa (**a**) or immunostained with the anti-sbIL−1β antibody (**b, c**). Note that IL−1β-immunopositive cells are only found in blood smears from infected fish. C control, I infected. Arrow indicates acidophilic granulocyte. Magnifications: **a** x20; **b, c** x100; inset x40.

Figure 4. a–c Blood acidophilic granulocytes from *V. anguillarum*-infected fish accumulate IL−1β intracellularly. Blood smears from infected fish were simultaneously stained with the G7 mAb (green), the anti-sbIL−1β antiserum (red) and DAPI (blue) and examined with a microscope using appropriate filters for green (**a**), red (**b**) and blue (**c**) fluorescence. Note that IL−1β immunostained cells coincide with the G7 immunostained cells. I infected. Magnification: **a, b, c** x20.

Figure 5. **a, b** Acidophilic granulocyte location is altered in the head-kidney of *V. anguillarum* infected fish. Head-kidney sections of control specimens (**a**) or following 16-h challenge with *V. anguillarum* (**b**) were immunostained with the G7 mAb. Note that acidophilic granulocytes occurred in clusters that often surround a blood vessel (V) in control head-kidneys, while they appeared isolated and scattered in infected head-kidneys. An asterisk indicates the melanomacrophage centre. Magnification: **a, b** x20.

Figure 6. Acidophilic granulocytes are mobilized from the head-kidney to the site of inflammation following *V. anguillarum* infection. Percentage of G7 immunoreactive cells (acidophilic granulocytes) in head-kidney (HK), peritoneal exudate (PE) and peripheral blood (B) of control specimens or following 16-h challenge with *V. anguillarum*. Data are presented as mean±SE of four fish. Asterisks denote statistically significant differences ($P<0.0001$) between control and infected fish.

FIGURE 1

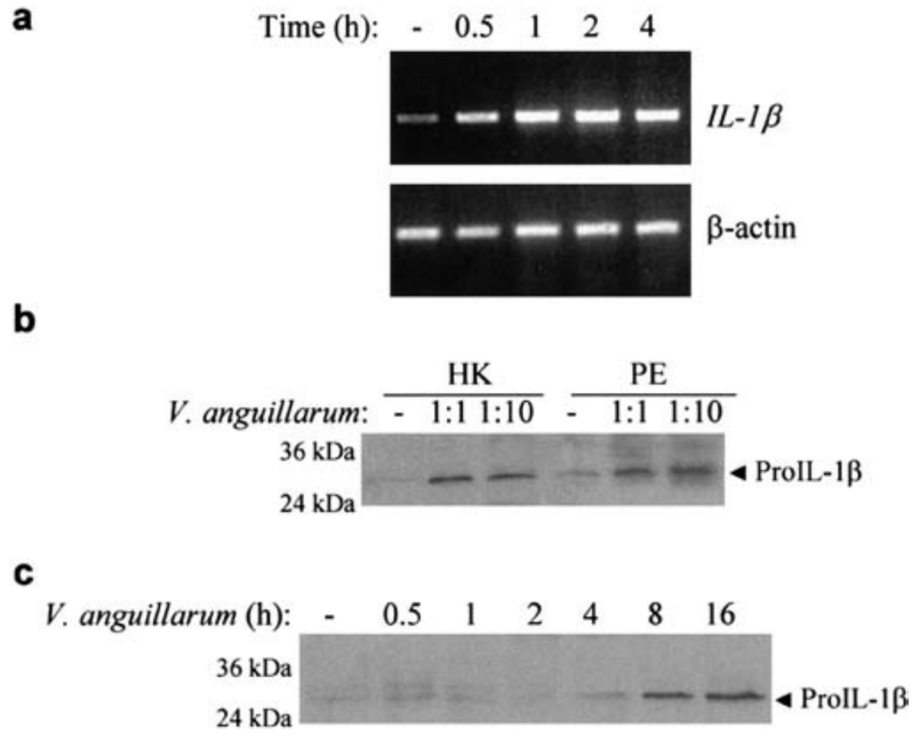

a
Time (h): - 0.5 1 2 4
IL-1β
β-actin
b
HK
PE
V. anguillarum: - 1:1 1:10 - 1:1 1:10
36 kDa
24 kDa
ProIL-1β
c
V. anguillarum (h): - 0.5 1 2 4 8 16
36 kDa
24 kDa
ProIL-1β

FIGURE 2

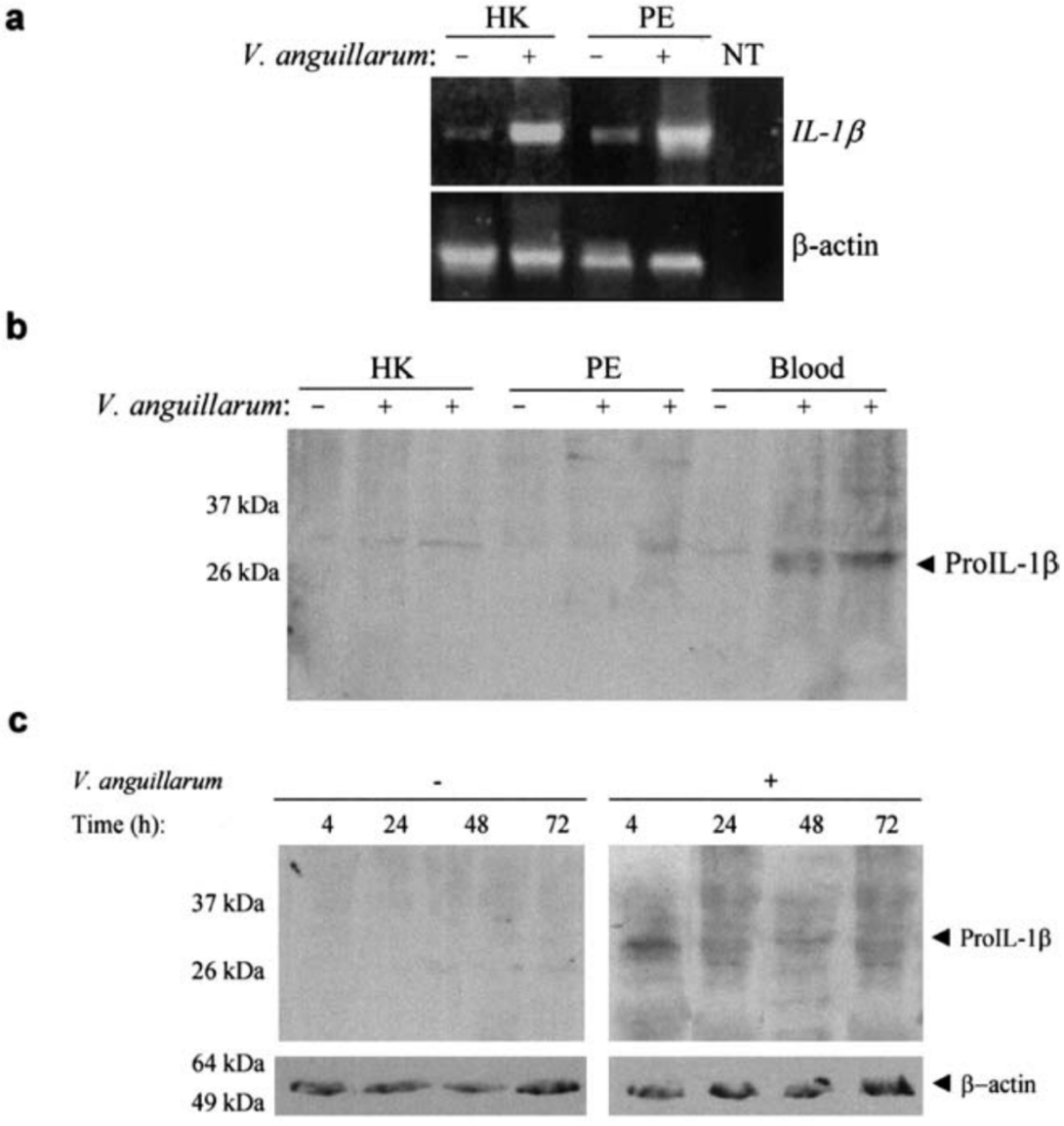

a
HK
PE
V. anguillarum: – + – + NT
IL-1β
β-actin
b
HK
PE
Blood
V. anguillarum: – + + – + + – + +
37 kDa
26 kDa
ProIL-1β
c
V. anguillarum
-
+
Time (h): 4 24 48 72 4 24 48 72
37 kDa
26 kDa
ProIL-1β
64 kDa
49 kDa
β–actin

FIGURE 3

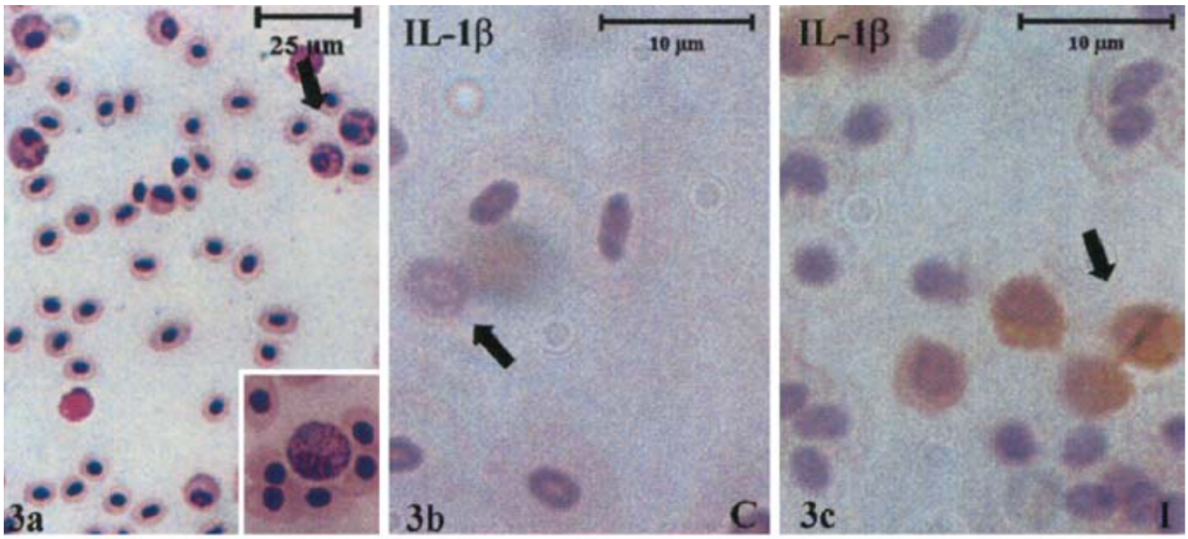


FIGURE 4

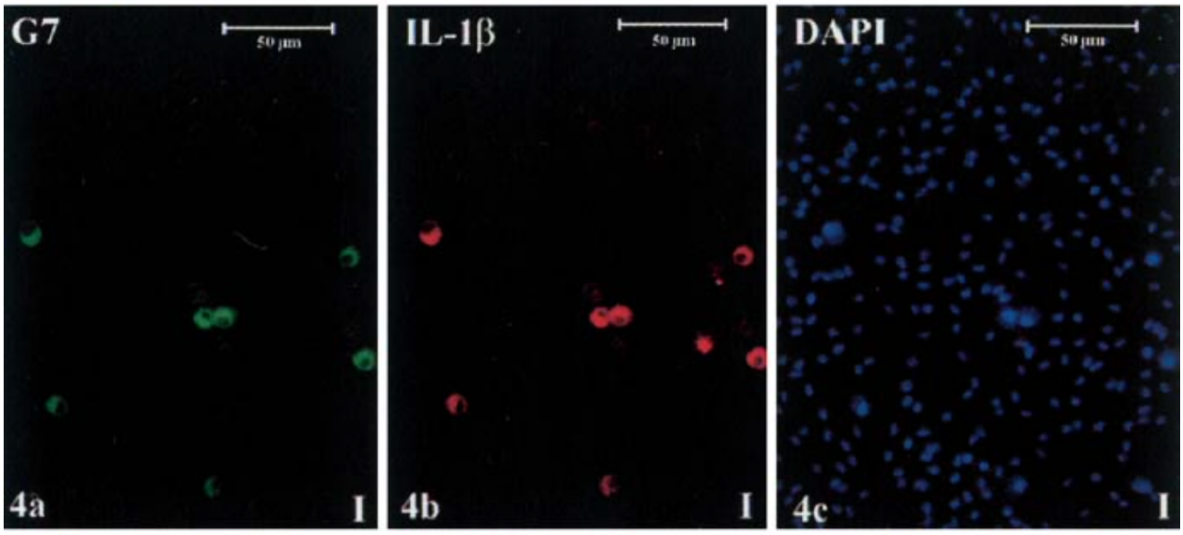


FIGURE 5

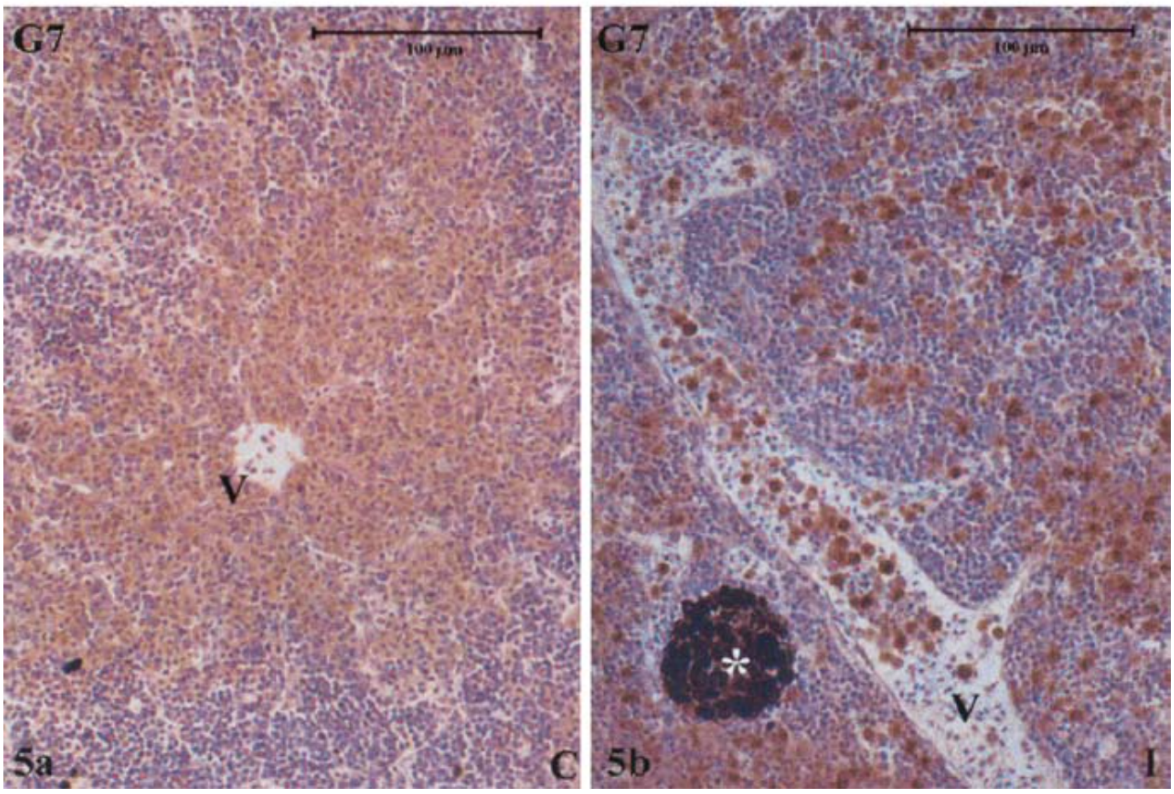
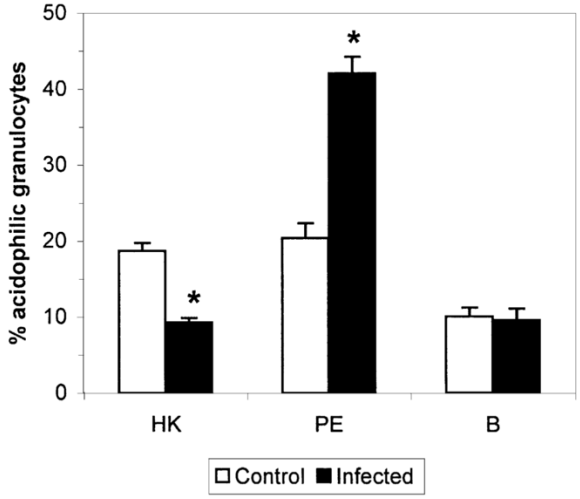


FIGURE 6


% acidophilic granulocytes
50
40
30
20
10
0
*
*
HK
PE
B
Control
Infected