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ORIGINAL ARTICLE

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Measuring some flounder (*Platichthys flesus* L.) innate immune responses to be incorporated in effect biomonitoring concepts

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Abstract For an implementation of innate immune responses of flounder (*Platichthys flesus*) in an integrated biological effect monitoring concept, leucocytes were isolated from peripheral blood, head kidney and spleen, and analysed for their capacity to mount a respiratory burst response upon phorbol ester stimulation. Responding cells were identified by reduced nitro-blue-tetrazolium salt deposits and by dihydro-rhodamine fluorescence in light microscope and flow cytometric analysis. Responding cells were found in head kidney derived cell suspensions rather than in peripheral blood or spleen. Parallel cytometric and microscopic analysis indicated that responding cells had a granulocyte or monocyte morphology, were alpha-naphtyl-esterase or myeloperoxidase positive and in flow cytometry exhibited a characteristic forward and side scatter (FSC/SSC) pattern. These cells represented 30–40% of head kidney derived cell suspensions and only 4–5 % of peripheral blood and spleen. In order to reduce sampling effort in field studies, leucocyte cell suspensions derived from flounder head kidney could be used in respiratory burst assays without further enrichment protocols. This paper combines, for the first time, conventional and cytometric analysis of phagocytes derived from flounder peripheral blood and head kidney.

Keywords Flounder leucocyte · Lysozyme · Respiratory burst

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Introduction

Innate immune responses of fish and invertebrates are discussed as promising candidates for biomarkers in an assessment of the impact of pollutants or xenobiotics on aquatic biota (Dunier and Siwicki 1993; den Besten 1998; Bols et al. 2001). Innate immune responses protect organisms against infection without depending upon prior exposure to any particular microorganism. This could mean that they directly reflect interactions of the species with other organisms. In addition, many components of innate immunity appear to be evolutionarily conserved (Hoffmann et al. 1999; Ulevitch 2000), which could indicate that the sensitivity of an innate immune mechanism to a particular contaminant is similar between species. This would make predictions of impacts of toxicants in the environment easier. Innate responses comprise biochemical and cellular processes, and in laboratory and field studies, many different parameters have been monitored [reviewed by Dunier and Siwicki (1993); Bols et al. (2001)]. Promising candidates for use in environmental studies were phagocytosis and production of reactive oxygen species (ROS) by phagocytic leucocytes (Bols et al. 2001).

Phagocytic leucocytes are present in almost all fish tissues and are regarded as a first line of defence against invading pathogens. For functional studies, both resident macrophages and circulating monocytes and granulocytes were isolated from various tissues such as gills (Lin et al. 1998), from the circulation, or from lymphoid tissues like head kidney or spleen (Secombes 1990). While the proportion of monocytes and granulocytes in the peripheral blood of healthy fish is only about 10%, these leucocyte populations are much more frequent in the head kidney (Scharsack et al. 2003). In healthy carp (*Cyprinus carpio*), approx. 30–40% of head kidney leucocytes were identified as granulocytes and about 20% as monocytes (Scharsack et al. 2003). These proportions can alter drastically under stress or infection (Ellis 2001). In stressed rainbow trout (*Oncorhynchus mykiss*) or in carp under haemoflagellate infection, an influx of granulocytes

into the peripheral blood was observed (Scharsack et al. 2001, 2003) which may result in a depletion of these cells in the head kidney. Monitoring changes in the composition of cellular subsets in the peripheral blood or lymphoid organs, however, is hampered by the lack of specific cell markers (Miller 1998). The identification of piscine leucocyte populations has to rely on morphological, cytochemical and functional characteristics. In numerous teleost species, cells with monocyte morphology exhibited strong esterase activity and granulocytes were positive for peroxidase activity (Rowley et al. 1988; Ainsworth 1992). In addition, leucocyte populations from the peripheral blood as well as from lymphoid tissues were analysed by means of flow cytometry and characterized according to their cytometric characteristics (Verburg-van Kemenade et al. 1994; Chilmonczyk et al. 1997; Scharsack et al. 2003). Thus, in this paper, attempts were undertaken to characterize responding cell populations from flounder according to their morphological, cytochemical and cytometric characteristics.

For the assessment of impacts of xenobiotics on cellular responses, the activity of phagocytic leucocytes such as neutrophilic granulocytes or monocytes was monitored in various studies (Zelikoff 1993; Secombes et al. 1997; Zelikoff et al. 1997). Phagocytosis is a complex process which comprises various steps: migration, adhesion, ingestion of particles, degranulation and respiratory burst by the production of ROS (English 1999). Most ecotoxicology studies focus on the ingestion of particles (phagocytosis) or respiratory burst, which can be measured by means of flow cytometry or in colorimetric assays (Secombes 1990; Chilmonczyk et al. 1997). The respiratory burst may be measured in several ways, which again can complicate comparison studies. In addition, cells can be collected from different organs and activated by various stimuli to trigger respiratory burst activity. As the respiratory burst appears to hold promise as a bioindicator for fish health (Rice et al. 1996) standardization of experimental protocols among research groups is considered to facilitate its use in environmental studies (Bols et al. 2001).

In an attempt to incorporate measurements of innate immune responses in an integrated biological effect monitoring concept in flounder (*Platichthys flesus* L.), leucocytes were isolated from peripheral blood, head kidney and spleen and analysed for the presence of different leucocyte subsets. Cells from these tissues were assessed for their capacity to mount a respiratory burst response upon stimulation by the synthetic agonist phorbol-12-myristate-13-acetate (PMA). The analysis of cellular subsets and respiratory burst response was done using conventional microscopic and colorimetric methods as well as flow cytometric analysis. Flow cytometry-based applications have been used to study various aspects of fish genetics and immunology (Thuvander et al. 1992, Verburg-van Kemenade et al. 1994) and were only recently applied in fish toxicology studies (Chilmonczyk et al. 1997).

Materials and methods

Fish

Flounder (*Platichthys flesus* L.) for this study were collected in the German Bight of the North Sea at a location in the Elbe estuary off Cuxhaven harbour during cruises with the research vessel '*Uthörn*' from the Alfred Wegener Institute. Sampling campaigns were conducted in April and September 1999 and 2000. Fishing was done with a bottom trawl (opening 1.5 m, mesh width in the cod end 40 mm stretched mesh). The fishing period was limited to 30 min to keep fish stress as low as possible. Onboard, the fish were sorted out immediately and kept in tanks with permanent seawater flow-through and aeration for up to 6 h until further processing took place. Only macroscopically healthy flounder of the size class 18–25 cm were used for this investigation. Some flounder were dissected onboard the research vessels, where blood and tissue samples were taken and processed in the laboratory immediately as described below. Some flounder were brought to the laboratory and kept in 80 l plastic aquaria with recirculated and aerated artificial seawater (Tropic Marin, Rebie, Bielefeld, Germany) at 14°C (±1.5) in the dark. The fish were fed daily ad libitum with frozen shrimps. All fish were acclimatized to laboratory conditions for 2 weeks before they were used for tissue sampling.

Examination procedure

Onboard the research vessel, body length and weight of each fish were measured and blood was drawn from the caudal vein into disposable syringes prefilled with a lithium-heparin bead (Sarstedt, Germany). The blood was transferred to centrifugation tubes and centrifuged at 2,000 *g* for 15 min at 4°C. Then the supernatant plasma was collected and frozen at −80°C. For flow cytometric analysis, blood was collected in the laboratory by caudal vein puncture into syringes prefilled with heparinised medium (RPMI medium, supplemented with 50,000 IU l^{-1} sodium heparin). Then the fish was killed and dissected, and head kidney and spleen were removed and transferred into centrifugation tubes filled with wash medium (RPMI medium supplemented with 10,000 IU l^{-1} sodium heparin; chemicals: Sigma, St. Louis, Mo.; medium: Biochrom, Berlin, Germany) and stored at 4°C for up to 24 h for further processing.

Leucocyte isolation

Media and cells were kept on ice and washing procedures were performed at 4°C. Peripheral blood leucocytes (PBL) were separated from erythrocytes by centrifugation (30 min, 750 *g*) over Lymphoprep (Nycomed, Oslo, Norway) as described by Miller and McKinney (1994). Cell suspensions of head kidney leucocytes (HKL) and spleen were prepared by forcing the tissues through a 100 µm nylon screen (Swiss Silk Bolting Cloth Mfg, Zürich, Switzerland). Isolated HKL, PBL and spleen leucocytes were washed three times with wash medium (10 min, 550 *g*) and resuspended in cell culture medium: RPMI-1640 supplemented with 1% [v/v] carp serum (serum from 15 individual *Cyprinus carpio* L. was pooled, heat inactivated for 30 min at 56°C, 0.2 µm filtered and stored at −20°C until use), 100,000 IU l^{-1} penicillin, 100 mg l^{-1} streptomycin and 4 mM L-glutamine (all chemicals: Biochrom). Numbers of viable cells were determined by trypan blue exclusion in a Neubauer haemocytometer.

For density gradient centrifugation of flounder HKL and PBL, a gradient was established by the centrifugation of 20, 40 and 60% Percoll solutions (Pharmacia, Sweden) at 30,000 *g* for 30 min. 3 ml of HKL or PBL suspension were layered on top of the gradient and centrifuged for 30 min at 750 *g*. Fractions were collected separately into centrifugation tubes and washed two times with wash media. The cells were adjusted to a density of 10^6 cells ml^{-1} and cytospin preparations were made for cytological staining and cytochemistry. In addition, samples of these cells were subjected to flow cytometric analysis.

To lyse erythrocytes in cell suspension of spleen or peripheral blood, the cells were exposed to distilled water for 5 s, 10 s, 15 s, 20 s, 25 s or 30 s. Then the cell suspensions were analysed by means of a flow cytometer. Nuclei of lysed erythrocytes were excluded from measurement and evaluation by appropriate adjustment of the threshold.

Production of ROS by flounder leucocytes

Generation of ROS by flounder leucocytes was measured by means of the intra-cellular oxidation of fluorescent di-hydrorhodamine (DHR 123) and by the nitro blue tetrazolium salt (NBT) reduction assay. For the DHR assay, PBL, HKL and spleen cells (10^6 cells ml^{-1}) were incubated for 15 min in cell culture medium with DHR (1 mg l^{-1}) and 0.15 mg l^{-1} phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, Germany) or without PMA in flow cytometer tubes in the dark. Morphology and fluorescence characteristics were recorded immediately after incubation, by means of flow cytometry. As the incubation of cells in the presence of PMA reduced the viability of granulocytes in a time-dependent manner, the incubation time of 15 min was observed sharply. In addition, live cells were observed with a fluorescence microscope (Zeiss-Axiophot, Zeiss, Germany), and micrographs were taken on Ilford Pan F 50 film. For the NBT assay, HKL were incubated in 96-well flat-bottom microtitre plates (10^6 cells in a final volume of 175 µl cell culture medium). All set-ups were made at least in triplicate. Receptor-independent ROS production was induced by adding 0.15 mg l^{-1} PMA. The indicator NBT was added at 1 g l^{-1}. Wells without PMA served to determine the spontaneous ROS generation of cells. After incubation for 2 h at 18°C, the supernatants were removed and the cells were fixed by adding 125 µl of 100% methanol. Each well was washed two times with 125 µl of 70% [v/v] methanol. Methanol was removed and the fixed cells were air dried overnight and stored in the dark for up to 2 weeks. The reduced NBT (formazan) was dissolved in 125 µl 2 M KOH and 150 µl DMSO per well (All chemicals: Sigma, St. Louis, Mo.). The optical densities were recorded with a spectrophotometer at 650 nm. Additional replicates with cells in NBT were taken for microscopic examination with a photo microscope (Zeiss-Axiophot, Zeiss, Germany).

Cytochemistry

Myeloperoxidase staining

Air-dried smears of HKL cell suspension were fixed for 30 s in a mixture of one part of 37% formalin to 10 parts of 95% ethanol, rinsed 2 min under tapwater and air dried in the dark (Kaplow 1965). Activity of myeloperoxidase was visualized by means of diaminobenzidine (DAB, Fluka-Chemie, Switzerland), which in the presence of H_2O_2 is oxidized by the enzyme myeloperoxidase and gives a brown pigment [Lojda et al. (1976) modified from Graham and Karnovsky (1966)]. Staining of nuclei was done with Harris' haematoxylin (Merck, Germany).

Nonspecific esterase activity

Air-dried smears from the HKL cell suspension were fixed in a formalin saturated atmosphere for 2 min (Lin et al. 1998). A modified staining method with α-naphthyl acetate (Sigma-Aldrich, Germany) according to Davis and Ornstein (1959) was used to detect esterase activity (Romeis 1989). According to this method, α-naphthyl acetate in the presence of freshly formed diazonium salt of pararosaniline is enzymatically hydrolysed by the esterase, liberating free naphthol compounds. These then couple with the diazonium salt to give a red deposit at the site of the enzyme. The nuclei were counterstained with Harris' haematoxylin solution (Merck, Germany).

In addition, smears were air dried, fixed with methanol for 3 min and stained with Giemsa (Merck, Germany).

Slides were observed with a photo-microscope (Zeiss-Axiophot, Zeiss, Germany), and microphotographs were taken using an Ilford, Pan F film.

Flow cytometric analysis of leucocytes

Cell suspensions of peripheral blood, head kidney and spleen were analysed with a flow cytometer (FACScan, Becton Dickinson, Germany, single excitation wavelength of 488 nm) immediately after isolation and after incubation with DHR. Plates with cultured cells were placed on ice (15 min), briefly shaken, then the whole content of each well was transferred to individual flow cytometer tubes, and 2 mg l^{-1} propidium iodide (Calbiochem, Bad Soden, Germany) was added to each tube to identify dead cells. Forward (FSC) and side scatter (SSC) characteristics of 10,000 events were acquired in linear mode, fluorescence intensities at wavelengths of 530 nm, and 650 nm were acquired at log scale. All flow cytometric data were analysed with the software WinMDI, version 2.8 (Trotter 1998). Cellular debris with low FSC characteristics and propidium iodide-positive, dead cells were excluded from further evaluation. Cell viability was monitored by means of propidium iodide uptake, and for each cell culture, the proportion of dead cells was calculated. In all experiments, cell viability was above 90%.

Statistics

Correlations between leucocyte numbers and ROS readings were calculated with Pearson's product moment correlation or with Spearman's rank correlation. Correlations were considered to be significant at $P<0.05$.

Results

Leucocyte typing and ROS production

Flow cytometric analysis of peripheral blood and spleen leucocytes of flounder yielded one main population with small FSC/SSC profiles (Fig. 1a, b, region 1, R1). In parallel microscopic evaluation, these cells showed the morphology of lymphocytes (Fig. 2). Cells from the head kidney exhibited a more complex pattern (Fig. 1c). Three major populations could be differentiated: cells with small FSC/SSC profiles in R1, a cell population with increased FSC/SSC characteristics in R2 and a third population with low SSC values but increased FSC values in R3 (Fig. 1c). In microscope analysis of head kidney-derived leucocytes, small lymphocytes with a condensed nucleus, and larger cells with an increased nucleus/plasma ratio were observed. In Giemsa-stained slides, these cells were identified as granulocytes and monocytes (Fig. 3). In addition, granulocyte-type cells showed a strong myeloperoxidase activity (Fig. 4) and cells with monocyte morphology were positive for α-naphthyl-esterase activity (Fig. 5).

In an attempt to analyse different leucocyte subsets from the head kidney for their ability to produce ROS, cells were incubated in the presence of NBT or DHR. Microscope observation revealed that granular cells had incorporated blue NBT formazan deposits (Fig. 6) and, upon DHR incubation, exhibited a clear green fluorescence (Fig. 7). Flow cytometric analysis (Fig. 8) showed that, in HKL suspensions, cells from the region R2 responded to PMA stimulation with ROS production. As

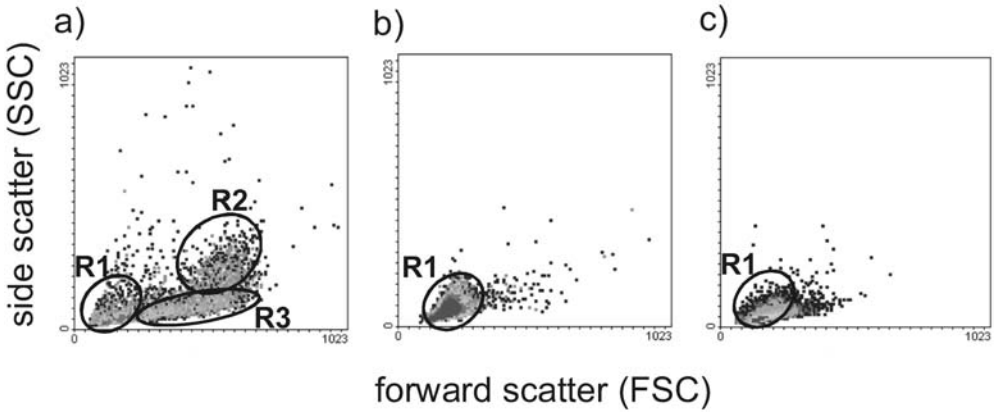
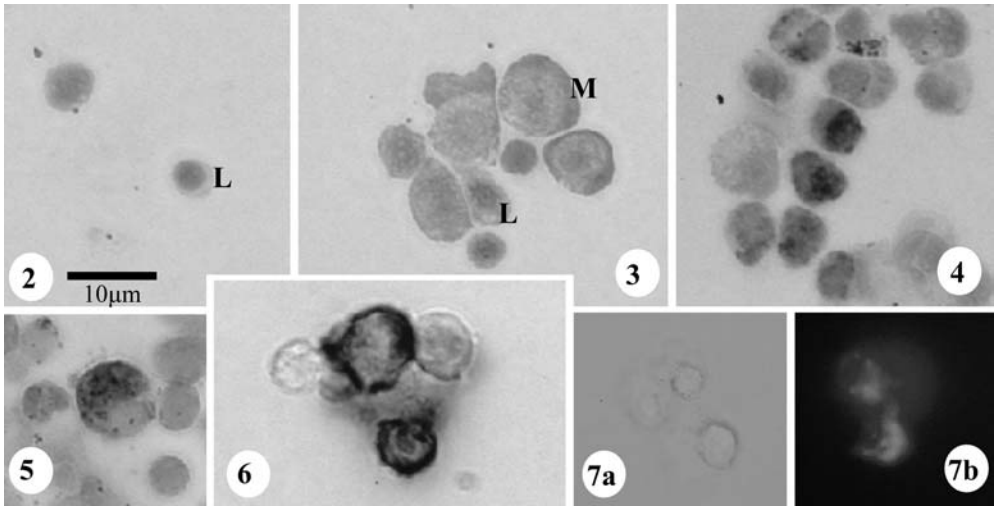


Fig. 1 Flow cytometric characteristics of flounder leucocytes from **a** head kidney, **b** peripheral blood and **c** spleen. Note that cell suspensions derived from peripheral blood and spleen contained only one cell population (region 1, *R1*; small cells with low FCS/SSC characteristics), whereas cells from the head kidney had a more complex pattern with cells with increased FSC/SSC characteristics (*R2*) and larger cells (*R3*). The figure shows flow cytometric characteristics from one individual as a representative out of 10 (spleen) or 40 (blood and head kidney) individuals with identical results



Figs. 2–7 *Platichthys flesus* leucocytes from peripheral blood and head kidney. Figure 2 shows peripheral blood derived lymphocytes and Figs. 3–7 head kidney-derived leucocytes

Fig. 3 Lymphocytes (*L*), monocytes (*M*) Giemsa stain

Fig. 4 Myeloperoxidase reaction

Fig. 5 A lpha-naphthyl-esterase reaction

Fig. 6 Nitro blue tretrazolium deposits in leucocytes

Fig. 7a, b Di-hydrorhodamine flourescence: **a** phase contrast image of three leucocytes; **b** the same cells under UV illumination: two cells show a clear fluorescence

ROS-production is a functional characteristic of granulocytes and monocytes (Secombes 1996), it can be concluded that these cells were found in the R2 region in flounder HKL suspensions.

Flow cytometric quantification of cellular subsets revealed that about 30% of cells in head kidney-derived leucocytes were from R2, compared to only 1.5–4.5% in peripheral blood or spleen (Table 1). Likewise, the numbers of ROS producing cells and spectrophotometric NBT readings in HKL were significantly higher than in PBL or spleen suspensions (Table 1). In cell suspensions, the number of ROS-producing cells as determined by DHR-fluorescence corresponded with the number of cells found in R2 (R=0.982, P<0.001, n=9). In addition, the number of DHR-fluorescent cells corresponded to parallel readings using the colorimetric NBT assay (R=0.719, P<0.001, n=18).

In an attempt to purify phagocytes from crude head kidney cell suspensions, HKL were centrifuged on continuous gradients prepared from 20, 40 and 60% Percoll gradients. After centrifugation on a 60% Percoll gradient, three populations were obtained, two major populations at a density of 1.05–1.06 kg l^{-1} and a third minor population at a density of 1.08–1.09 kg l^{-1}. Flow cytometric and microscope analysis showed that lymphocyte, granulocyte and monocyte type cells were present in all three populations. While population 1 mainly consisted of lymphocytes (50–60% of cells in region R1),

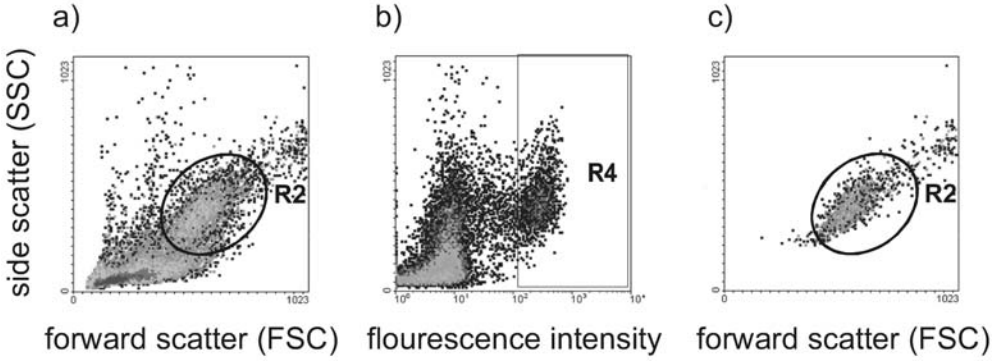


Fig. 8a–c Flow cytometric detection of respiratory burst activity in head kidney derived leucocytes. **a** FSC/SSC scatter diagram of a head kidney cell suspension. **b** Green fluorescence/SSC scatter diagram of the same cells after incubation with di-hydrorhodamine and stimulated with the phorbol ester PMA. Measurements gated in R4 were considered as fluorescent positive, which indicated respiratory burst activity. **c** FSC/SSC profiles of green fluorescence positive cells from the region R4 are characteristic for cells from the region *R2* in Fig. 1. The figure shows flow cytometric characteristics of head kidney derived leucocytes from one individual as a representative out of 10 with similar results

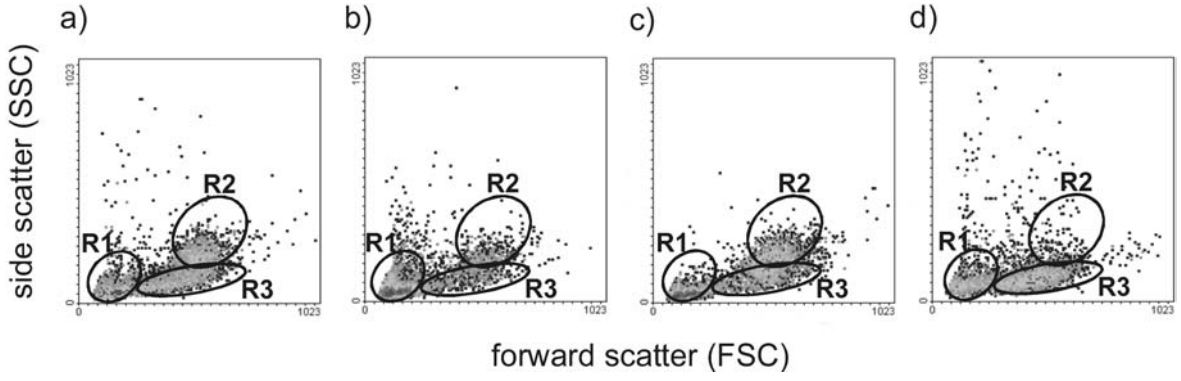


Fig. 9a–d FSC/SSC profiles of head kidney cells after centrifugation on a continuous gradient generated from a 60% Percoll solution. **a** Freshly isolated suspension. **b** Population 1, at a density of 1.05 kg l^{-1}. **c** Population 2 at a density of approx. 1.06 kg l^{-1}. **d** Population 3 at a density of approx. 1.08 kg l^{-1}. Population 3 contained a high number of erythrocytes and was red in colour. The figure shows flow cytometric profiles of head kidney cells from one individual fish out of a set of three individuals which gave the same results

Table 1 *Platichthys flesus*, proportion of leucocyte subsets and ROS production in lymphoid organs. Cells were characterized in flow cytometric analysis by FSC/SSC scatter profiles according to Fig. 1. Cells from region R1 were characterized by low FSC (cell size) and SSC (cell complexity) profiles, while cells from region R2 had increased FSC/SSC characteristics (see Fig. 1). The ROS production of corresponding cell suspensions was measured by means of the colorimetric NBT assay and the percentage DHR-fluorescence positive cells measured by flow cytometry. Data show mean and standard deviation of measurements from four individual flounder. Significant differences in cell distribution and ROS production were found between head kidney and peripheral blood and spleen measurements.

Tissue	% cells in the gate		NBT (O_2^-) (optical density)	DHR (H_2O_2) (% pos. cells)
	R1	R2		
Head kidney	52.4 (±2.7)	27.8 (±3.3)	0.320 (±0.109)	41.5 (±11.1)
Peripheral blood	90.4 (±4.3)	4.4 (±2.2)	0.143 (±0.101)	4.5 (±3.3)
Spleen	93.9 (±2.5)	1.5 (±0.6)	0.033 (±0.019)	1.5 (±1.3)

population 2 was rich in granulocyte/monocyte type cells (50–60% of cells in region R2, Fig. 9). Population 3 consisted of a ratio of 50–55% of cells from R1 and 40–45% of cells from R3 (n=3, Fig. 9). In cell lysis experiments, cells from R3 were identified as erythrocytes (not shown).

Discussion

European flounder (*Platichthys flesus*) is frequently used in biomonitoring studies conducted in coastal environments of northern Europe (Roose et al. 1998; Broeg et al. 1999; Grinwis et al. 2000). In these waters, the flounder is

widely distributed, lives in close contact with the sediment and is tolerant of changes in salinity which enables the fish to invade estuaries as well. Studies on flounder focus on chemical (Grinwis et al. 2000), pathological and parasitological (Broeg et al. 1999) analysis but also comprise measurements of innate immune parameters (Pulsford et al. 1995; Boonstra et al. 1996; Grinwis et al. 1998). Among these, serum lysozyme activity and responses of phagocytic cells were most promising candidates for biomarkers. These parameters were found to be susceptible to xenobiotics and can be measured in high throughput, inexpensive assays (Bols et al. 2001).

For an implementation of these parameters in an environmental monitoring study, we here analysed the capacity of flounder leucocytes for respiratory burst activity. NBT-formazan deposits as an indicator of intracellular secretion of superoxide anion were observed in leucocytes with granulocyte or monocyte morphology. These cells also exhibited myeloperoxidase activity, which was found to be characteristic of granulocytes in many fish species (Hine et al. 1987; Lehmann et al. 1994). In flow cytometric analysis, only cells with increased FSC/SSC pattern exhibited respiratory burst activity. In carp (Verburg-van Kemenade et al. 1994) and rainbow trout (Chilmonczyk et al. 1997), cells with this FSC/SSC pattern and these functional characteristics were regarded as granulocytes and monocytes. As in rainbow trout and carp (Chilmonczyk et al. 1997; Scharsack et al. 2001), head kidney-derived leucocytes of flounder contained increased proportions of cells with granulocyte or monocyte morphology and also showed increased respiratory burst activity. In peripheral blood and spleen, phagocytic cells were present in small numbers: cell suspensions from these tissues gave only low respiratory burst measurements in response to phorbol ester stimulation. In field studies, collection of PBL is less invasive but, due to the low signal obtained, their respiratory burst activity is difficult to measure. In addition, a neutrophil influx into the peripheral blood is observed upon acute stress (Chilmonczyk et al. 1997; Scharsack et al. 2001) or infection (Scharsack et al. 2000), which will cause an increase in respiratory burst activity and thus interfere with pollution-mediated effects. In head kidney, stress- or infection-related effects on proportion and activity of neutrophils can also be noticed when their respiratory burst activity is assessed (Chilmonczyk et al. 1997). Upon infection, most prominent effects were seen in basal ROS production (Scharsack et al. 2000), which is, however, more likely to represent routine metabolic activity of cells than an immune response (Turrens and Boveris 1980). Thus it might be concluded that measurements of basal ROS production or calculation of ROS stimulation indices have no major relevance in pollution monitoring studies.

Enrichment of ROS producing cells from crude head kidney cell suspensions occurred using density gradient centrifugation. Crude suspensions contained 30–40% of ROS producing cells. After density gradient centrifugation, this proportion increased to up to 60%. In common protocols for isolation of macrophages or granulocytes (Secombes 1990), density gradient centrifugation is followed by adhesion of cells to culture vessels and non-adherent cells will be washed away. Attachment to surfaces, however, is a step in the process of phagocytosis (English 1999), which could also be modulated by xenobiotics. In experiments where the ability of cells to attach to surfaces is impaired by xenobiotics, these cells would be lost during washing procedures, and respiratory burst activity would be measured only from those cells which retained their ability to attach to surfaces. Consequently, the signal might be too low because a significant number of cells would be lost, or it might be too high because the purification procedure selected for less affected cells. To avoid losses of non-adherent ROS-producing cells, we measured respiratory burst activity of cells in suspension. In order to minimize sampling effort for application in a field study, crude cell suspensions were used. We are aware that this procedure does not allow an adjustment of respiratory burst measurements according to phagocyte numbers and might result in a high variation of readings. Flow cytometric evaluation of ROS production allows an identification and quantification of responding cells. In addition to cell function, it allows a simultaneous observation of cell morphology and changes in the proportion of cellular subsets, but it relies on the analysis of live cells (Scheffold and Radbruch 1998). The FSC/SSC characteristics of head kidney derived flounder leucocytes changed after formalin fixation and storage, which did not allow morphometric analysis of cells at a later date (data not shown). The colorimetric NBT assay has to be performed with live cells as well. Methanol-fixed, air-dried plates could be stored for later analysis.

In conclusion, respiratory burst active phagocytes could be isolated from the head kidney of flounder in sufficient numbers to give good readings of ROS production in flow cytometric and colorimetric assays. The NBT assay and phorbol-ester stimulation of the cells gave reliable results. In addition, isolation and processing of the cells could be implemented in a work schedule for an integrated programme in environmental monitoring.

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