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Scavenger-receptor-mediated endocytosis of lipopolysaccharide in Atlantic cod (*Gadus morhua* L.)

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Summary

The mechanism of elimination of blood-borne *Vibrio salmonicida* lipopolysaccharide (LPS) from Atlantic cod (*Gadus morhua* L.) was studied. The anatomical distribution of LPS was determined using both morphological and radiotracing methods. Immunohistochemistry performed on tissue specimens after injection of LPS disclosed that the endocardial endothelial cells (EECs) represented the cellular site of uptake in heart. Co-injection of trace amounts of [^{125}I]LPS together with excess amounts of formaldehyde-treated albumin (FSA), a ligand for the scavenger receptor, significantly inhibited the accumulation of the radiotracer in heart only. Studies on purified monolayer cultures of atrial EECs showed that fluorescein-labelled LPS was taken up in structures reminiscent of endosomal/lysosomal vesicles. Incubation of cultures with [^{125}I]LPS together with excess amounts of FSA, fucoidan and dextran sulphate, molecules known to compete for endocytosis *via* the scavenger receptor, reduced uptake of the probe by 80 %. Mannan, a ligand for the mannose receptor, did not compete for uptake. Kinetic studies on the uptake and degradation of [^{125}I]LPS in cultured atrial endocardial cells revealed no degradation after 48 h of culture. In conclusion, we have shown that the EECs of cod remove *V. salmonicida* LPS from the circulation by scavenger-receptor-mediated endocytosis.

Key words: lipopolysaccharide, scavenger receptor, endocytosis, endothelium, endocardium, Atlantic cod, *Gadus morhua*.

Introduction

Lipopolysaccharides represent characteristic components of the cell envelope of gram-negative bacteria. The lipopolysaccharide (LPS) molecule is composed of three structural units; the O-polysaccharide, a core oligosaccharide and a lipophilic lipid A component. The lipid A portion represents the endotoxic component of LPS and is responsible for the typical endotoxin effects observed in mammals, such as fever, haemodynamic changes and disseminated intravascular coagulation and shock (Raetz, 1990; Rietschel and Brade, 1992). The toxic effect of LPS is explained by the fact that, in several mammalian species, LPS triggers macrophages to produce powerful inflammatory mediators such as tumour necrosis factor alpha (TNFα), interleukin-1α (IL-1α), IL-1β and IL-6. The increased production of these mediators is believed to be the direct cause of LPS toxicity (Callery et al., 1990; Chensue et al., 1991). Despite the fact that LPS is highly immunogenic in fish (Hastings and Ellis, 1990) and that LPS stimulates fish leukocytes *in vitro* and *in vivo* (Clem et al., 1985), it has been reported that endotoxic shock does not occur in fish (Berczi et al., 1966). The reason for these differences in LPS sensitivity between fish and many mammalian species is unknown.

Studies performed in rabbit and rat have shown that circulating LPS is mainly distributed to the liver (Mathison and Ulevitch, 1979). The liver macrophages (Kupffer cells) are largely responsible for this clearance. Sinusoidal liver endothelial cells (sLECs) and parenchymal cells contribute to a lesser extent (Ruiter et al., 1981; van Oosten et al., 1998). Kupffer cells, the largest population of macrophages in mammals, are not found in cod liver (Morrison, 1987). Nevertheless, the macrophages of fish, including cod, are thought to be important cells in disease resistance. They are phagocytic both *in vivo* (Ferguson, 1984) and *in vitro* (Braun-Nesje et al., 1981) and are considered to be the principal phagocytic cell in fish (Blazer, 1991). Fish macrophages are also known to act as accessory cells in specific immune responses (Clem et al., 1985; Miller et al., 1985) and to participate in the regulation of immune responses by both producing (Ellsaesser and Clem, 1994) and responding to (Francis and Ellis, 1994; Graham and Secombes, 1990;

Secombes, 1987) cytokine-like substances. Intravenous injection of S-type LPS into Atlantic cod (Dalmo et al., 1998) demonstrated that LPS is taken up by the endocardial endothelial cells (EECs) of the heart.

The EECs lining the muscular trabecula of Atlantic cod heart represent a general vertebrate reticuloendothelial, or scavenger, cell type with the capacity to clear and degrade a number of physiological and foreign waste macromolecules from the circulation by receptor-mediated endocytosis. Functional studies indicate that cod EECs express a set of at least four types of endocytic receptors for this purpose: (i) the collagen receptor (Koren et al., 1997; Smedsrød et al., 1995), (ii) the hyaluronan receptor (Seternes et al., 2001), (iii) the mannose receptor (Sørensen et al., 2001) and (iv) the scavenger receptor (Sørensen et al., 1998).

Both mannose and scavenger receptors are considered to be involved in host defence mechanisms. The mannose receptor mediates endocytosis and phagocytosis of glycoproteins and particles containing terminal D-mannose, L-fucose and/or *N*-acetyl-D-glucosamine (Ezekowitz and Stahl, 1988). These residues are abundant at the terminal position of yeast and bacterial cell wall glycoproteins (Ezekowitz et al., 1990; Ezekowitz and Stahl, 1988). The scavenger receptors are involved in host defence as pattern recognition receptors. Because of their ability to bind to negatively charged macromolecules, they recognise surface constituents of both gram-positive and gram-negative bacteria as well as the intact bacteria (Dunne et al., 1994; Hampton et al., 1991). By expressing both mannose and scavenger receptors, the EECs may play a role in the innate immune defence system in cod in addition to clearing waste molecules from normal tissue turnover.

The bacterium *Vibrio salmonicida* is highly pathogenic in cod and salmon and occasionally causes significant losses in the Norwegian aquaculture industry. The dominant antigens of *V. salmonicida* are a 24 kDa surface protein and the LPS molecule (Bøgwald et al., 1990, 1991). This LPS molecule is a so-called rough type (R type), composed of lipid A and an oligosaccharide moiety (Edebrink et al., 1996). The aim of the present study was to determine whether EECs take up *V. salmonicida* LPS *in vivo* and *in vitro* and to investigate whether the scavenger receptor of the EECs is responsible for the endocytosis of this molecule.

Materials and methods

Chemicals and media

Carrier-free Na[^{125}I] was purchased from the Institute of Energy Technology (Kjeller, Norway). 5-(4,6-Dichloro-triazine-2-yl) amino fluorescein hydrochloride (DTAF), Sephadex G-25 (PD-10, disposable column) and Percoll density medium were obtained from Pharmacia (Uppsala, Sweden). Broad-range prestained molecular mass standards were obtained from Bio-Rad laboratories (Richmond, CA, USA). Leibovitz 15 (L-15) medium was obtained from Gibco (Life Technologies, Paisley, Scotland), adjusted to 380 mosmol l^{-1} tonicity using 0.5 mol l^{-1} NaCl and supplemented with 0.33 g l^{-1} glucose and 0.05 g l^{-1} gentamycin. Foetal calf serum was purchased from Hyclone (Logan, UT, USA), and heparin was purchased from Novo Nordisk (Copenhagen, Denmark). RPMI 1640, supplemented with L-glutamine (2 mol l^{-1}), gentamicin (200 μg ml^{-1}) and fungizone (50 μg ml^{-1}), were purchased from Flow Laboratories (Irvine, Scotland, UK). Human fibronectin was a kind gift from Berit Hansen, University of Tromsø. Human serum albumin was purchased from Octapharma (Wien, Austria), and trypsin (1:250) was purchased from Difco (Detroit, MI, USA). Monoclonal antibodies against *V. salmonicida* LPS (7F3 and 2B5) were a gift from Sigrun Espelid (Norwegian Institute of Fisheries and Aquaculture Ltd, Tromsø, Norway). All other chemicals, unless stated otherwise, were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Animals

Fish for *in vivo* studies, Atlantic cod (*Gadus morhua* L.) (0.1–1.5 kg) were obtained from Tromsø Aquaculture Station (Norwegian Institute of Fisheries and Aquaculture Ltd, Tromsø, Norway). The fish were kept in plastic tanks (2000 l) supplied with running sea water at 8 °C. All fish were adapted to the test conditions for at least 1 week before the experiments started and were fed a commercial diet daily before and during the adaptation and experimental period. For cell isolation, hearts from net-pen-captured Atlantic cod (1–4 kg) were used. The fish were kept in large dip nets in the sea (3–10 °C) and fed a commercial diet. Male Sprague-Dawley rats, purchased from Charles River, Wiga, Germany, and fed a standard diet, weighed 200–250 g when used in experiments.

Ligands for endocytosis studies

Formaldehyde-treated albumin (FSA) was prepared as described by Mego et al. (1967). Mannan, fucoidan and dextran sulphate were purchased from Sigma. Lipopolysaccharide from *V. salmonicida* was isolated according to the phenol:chloroform:petroleum ether method for R (rough) LPS of Galanos et al. (1969).

Preparation of [^{125}I]LPS

V. salmonicida LPS was modified and labelled with Na[^{125}I] as described previously (Ulevitch, 1978). Briefly, the LPS primary amino groups were coupled to benzamidate esters (ρ-OH methylbenzimidate) at alkaline pH. The resulting LPS derivative was radiolabelled with carrier-free Na[^{125}I] in a direct reaction using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen; Pierce, Rockford, IL, USA) as oxidising agent according to instructions provided by the manufacturer. Free iodine was removed from the solution by gel filtration on a PD-10 column eluted with phosphate-buffered saline (PBS). The specific activity of the [^{125}I]LPS was determined to be 3×10^6 cts min^{-1} mg^{-1}. Radioactivity was measured using a Packard gamma counter (Packard Instrument Company, IL, USA). SDS–PAGE and subsequent western

blotting confirmed the purity of the [^{125}I]LPS and its reactivity against antibodies.

Preparation of fluorescein-labelled LPS

Fluorescein-labelled LPS (F-LPS) was prepared as described previously (DeBelder and Granath, 1973). In brief, LPS (60 mg) from *V. salmonicida* was solubilised in borate-buffered dimethylsulphoxide (50 %; pH 9.8) to give a final concentration of 10 mg ml^{-1}. DTAF (0.5 mg mg^{-1} LPS) was then added, and the reaction mixture was stirred for 48 h at room temperature (22 °C) in the dark. The mixture was then extensively dialysed against 50 % dimethylsulphoxide for 2 days and thereafter against distilled water for 10 days. Finally, isolation of F-LPS was accomplished by Sephadex G-25 gel chromatography (PD-10). Its purity and reactivity against antibodies were confirmed by SDS–PAGE and subsequent western blotting.

SDS–PAGE and western blotting

SDS–PAGE (Laemmli, 1970) was performed in a Bio-Rad mini-PROTEAN II electrophoresis cell using 10 % Ready Gels from Bio-Rad (40 min at 200 V). Electrophoresed samples were blotted electrophoretically to a Millipore PVDF membrane using a Bio-Rad Mini Transblot electrophoretic transfer cell (100 V for 1 h at 4 °C). Blots were blocked in 3 % non-fat dry milk in 0.02 mol l^{-1} Tris-HCl buffer at pH 7.5 containing 0.5 mol l^{-1} NaCl and 0.3 % Tween 20 (TTBS) for 2 h at 22 °C, and then incubated with monoclonal antibodies (7F3 and 2B5) reported to be specific for *V. salmonicida* LPS (Bøgwald et al., 1990; Espelid et al., 1987) in TTBS containing 3 % non-fat dry milk at 4 °C overnight. The membranes were washed four times with TTBS and then incubated with a 1:10 000 dilution of rabbit anti-mouse immunoglobulins conjugated with alkaline phosphatase (DAKO AS) in TTBS for 1 h at 22 °C. The membranes were then washed four times with TTBS and twice in CDP-Star assay buffer (New England BioLabs, Beverly, MA, USA). Membranes were incubated with 1:500 CDP-Star substrate (New England BioLabs, Beverly, MA, USA) in assay buffer for 5 min and exposed to autoradiography film. Band densities on the developed film were measured using a Stratagene Eagle Eye II still video system.

Biological activity of native and modified LPS

The biological activity of purified native and modified *V. salmonicida* LPS was measured as its ability to induce TNFα production by murine peritoneal macrophages. Balb/c male mice (Harlan, UK), 6–8 weeks old, were killed by CO_2 suffocation. Resident peritoneal cells were harvested by lavage with 5 ml of ice-cold RPMI-1640 medium with synthetic serum replacement (SSR-II, Medicult, Copenhagen, Denmark). Cells were seeded at a density of 2×10^5 cells per well into 96-well plates (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA; catalog no. 353072) and cultured at 37 °C in a humidified atmosphere containing 5 % CO_2. After 2 h, non-adherent cells were washed off, and the adherent cells were cultured in 200 µl of SSR-II medium overnight. Next morning, fresh medium was added and the cells were incubated with *V. salmonicida* LPS (1 µg ml^{-1}) in its native form, with F-LPS or an LPS derivative or with *Escherichia coli* LPS (O26:B6 LPS; Difco Laboratories Inc. Detroit, MI, USA). Supernatants were collected 24 h after stimulation. Unstimulated cultures served as controls. The concentration of TNFα in the cell culture supernatants was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit purchased from Biosource Europe, S.A. (Nivelles, Belgium).

Anatomical distribution

Twelve cod were anaesthetised by immersion in 0.004 % (w/v) benzocaine solution and injected intravenously with trace amounts of [^{125}I]LPS (0.5–1 µg kg^{-1} body mass) in a total injection volume of 200 µl of PBS per fish. At specified times, blood samples were collected from the caudal vein approximately 2 cm caudal to the injection site. The fish were killed by a blow to the head immediately after blood sampling, and the heart, anterior kidney, spleen, liver, intestine and blood were removed and analysed for radioactivity in a Packard gamma counter. The carcass of the fish, including the head, gills, muscle tissue and skin, was placed in a single tube and analysed for radioactivity. Parallel series of three fish were sampled 1, 4, 24 and 48 h after administration. As a control for accurate intravenous injection, the tissue at the injection site was excised and radioactivity was quantified in all fish. Total blood mass was taken as 5 % of body mass (Sørensen et al., 1998).

Inhibition studies were performed by co-injecting intravenously trace amounts of [^{125}I]LPS (0.5–1 µg kg^{-1} body mass) with unlabelled FSA (5–10 mg kg^{-1} body mass) in a total injection volume of 500 µl of PBS per fish. Sixteen cod were used in this experiment; eight were injected with [^{125}I]LPS/FSA and eight with [^{125}I]LPS only. After 1 h, blood samples were collected from the caudal vein approximately 2 cm caudal to the injection site. The fish were killed by a blow to the head immediately after blood sampling. The heart, anterior kidney and liver and blood samples were removed and analysed for radioactivity in a Packard gamma counter.

Uptake of LPS by blood cells was investigated by density gradient sedimentation of whole blood after intravenous injection of [^{125}I]LPS. Four cod were anaesthetised and injected intravenously with [^{125}I]LPS (0.5–1 µg kg^{-1} body mass) in a total injection volume of 600 µl of PBS per fish. Blood samples were collected (as described above) in heparin-coated tubes 1 h and 24 h after injection. A discontinuous gradient was made up of 4 ml of 34 % and 4 ml of 48 % Percoll density medium. Whole blood (2 ml) was loaded onto each density gradient for centrifugation at 400 ***g*** for 35 min at 4 °C in a centrifuge equipped with a swing-out rotor (Kubota 8800, Kubota Corporation, Tokyo, Japan). After centrifugation, 10 fractions of 1 ml were collected from the gradients. Fractions 1–4 contained plasma, fractions 5–7 contained the 34–48 % interface where leukocytes were trapped, and fractions 7–10 contained red blood cells. The fractions were analysed for radioactivity in a Packard gamma counter.

Immunohistochemistry

Vibrio salmonicida LPS was detected by immunohistochemistry 48 h after intravenous injection of LPS (2 mg kg^{-1} body mass) into Atlantic cod. Paraffin sections were dewaxed in xylene and a graded series of alcohol concentrations and then hydrated by immersion in distilled water. The sections were then unmasked by boiling in 0.1 mol l^{-1} citrate buffer, pH 6.0, for 2–5 min in a microwave oven or by treating them with trypsin (Gibco; 1:250) in Tris-buffered saline (TBS) for 10 min at room temperature. All the subsequent incubations were carried out at room temperature. After two washes of 10 min in TBS, the sections were incubated in TBS containing 30 % dried milk for 20 min and then in 7F3 monoclonal antibody (against *V. salmonicida* LPS), diluted 1:20 000, for 30 min. After rinsing in TBS, sections were incubated for 30 min with a biotinylated secondary antibody (rabbit anti-mouse Ig; DAKO AS, Glostrup, Denmark) and thereafter with avidin/biotin/alkaline phosphatase complex (Vectastain ABC-AP stain, Vector Laboratories Inc., Burlingame, Canada). Reaction products were visualised with Fast Red TR/naphthol AS-MX. Sections were counterstained with haematoxylin and mounted in Aquamont (Kebo Lab AS, Oslo, Norway). As a control for non-specific background staining, the primary antibody was omitted. Sections from fish not injected with LPS were used as a control for non-specific tissue binding of the primary antibody.

Isolation and cultivation of atrial EECs (aEECs)

Functionally intact aEECs from cod were purified according to Koren et al. (1997). In brief, the heart was dissected out and perfused with L-15 medium containing heparin (10 i.u. ml^{-1}). The atria were dissected free and cut open. Ostial tissue rich in fibrocytes and macrophages was discarded, and the remaining tissue was transferred to a 50 ml sterile plastic tube with 25 ml of calcium-free buffer (Smedsrød and Pertoft, 1985). After a 30 min incubation with horizontal shaking (250 cycles min^{-1}), the buffer was changed. The atria were then incubated with the following solutions: trypsin (0.5 mg ml^{-1}) and EDTA (0.1 mg ml^{-1}) in PBS for 5 min followed by collagenase (0.5 mg ml^{-1} in L-15 medium supplemented with 0.7 mg ml^{-1} $CaCl_2 \cdot 2H_2O$) for 30 min. The contents of the tube were then transferred to a sterile Petri dish, and the cell suspension thus obtained was used to flush the atrium several times with the jet from a 10 ml plastic syringe. The remaining tissue was discarded, the cell suspension was centrifuged for 5 min at 400 ***g*** and the pellet was resuspended in L-15. Contaminating macrophages (adherent cells) were removed according to the method of Sørensen et al. (1998). The non-adherent cells were seeded on plastic (Falcon, Becton Dickinson & Company, NJ, USA) or glass tissue culture slides precoated with fibronectin (0.5 mg ml^{-1}). The incubation medium was L-15 supplemented with 10 % foetal calf serum. The isolation procedure and incubations were carried out at 12–14 °C. The cells were washed with L-15 medium after 24 h and used in experiments the same day or the next day. The number of cells seeded per 2 cm^2 was approximately 10^6. Contamination by cardiomyocytes and a few macrophages was observed. On average, more than 90 % of the cells were aEECs, as evaluated by morphological characteristics.

Isolation of rat liver sinusoidal endothelial cells (sLECs)

Rat sLECs were prepared as described previously (Smedsrød et al., 1985). Briefly, rat livers were perfused with collagenase, and the resulting suspension of single cells was subjected to low-speed centrifugation followed by sedimentation in Percoll gradients. The resulting non-parenchymal cells were suspended in serum-free RPMI 1640 tissue culture medium and seeded in dishes (for endocytosis studies) or on coverslips (for morphology studies) coated with fibronectin by exposing the growth surface for a few seconds to a solution of 0.5 mg ml^{-1} fibronectin. After initial attachment and washing, the resulting cultures contained more than 90 % sLECs.

Endocytosis of LPS by cultured cod aEECs and rat LECs

Atrial EEC cultures established in 2 cm^2 wells (approximately 3×10^5 cells attached and spread per cm^2) were washed several times with L-15 medium and supplied with fresh medium containing 1 % human serum albumin (HSA) and trace amounts of [^{125}I]LPS (20×10^3 cts min^{-1}) in a total incubation volume of 200 µl per well. Incubations of [^{125}I]LPS at 12 °C were terminated after various times by removing the incubation medium and washed once with 500 µl of PBS. The incubation medium was then transferred to a PD-10 column and eluted with PBS. Fractions of 0.5 ml were collected. Intact ligand eluted in the void volume and degraded ligand eluted in the total volume. Cell-associated ligand was quantified by solubilising the cell layer with 1 % SDS, followed by counting in a gamma counter. Intracellular degradation was determined by solubilising cells after a 48 h continous incubation with trace amounts of [^{125}I]LPS in 1 % SDS in PBS. The solubilised cells were then transferred to a PD-10 column and eluted with PBS. Fractions of 1 ml were collected. Eluted fractions were analysed in a gamma counter.

The effect of LPS on aEEC viability was investigated in culture. Cultures of aEECs were incubated with or without 10 µg ml^{-1} native *V. salmonicida* LPS for 0, 24 or 48 h at 12 °C. The reduction in cell viability during the experimental period was measured by staining cell nuclei for 30 min with 0.1 % Crystal Violet after a 15 min fixation in 1 % glutaraldehyde (Gillies et al., 1986). The amount of dye was quantified by solubilising the absorbed dye in a 0.2 % solution of Triton X-100 and determining optical density. The absorbance was measured at 590 nm.

Receptor-specific endocytosis of [^{125}I]LPS was examined by inhibition studies. Monolayer cultures were incubated for 2 h with trace amounts of ^{125}I-labelled ligand alone (control) or together with excess amounts of unlabelled macromolecules (100 µg ml^{-1}). Endocytosis experiments were terminated after

2 h at 12–14 °C by removing the incubation medium and one washing volume of 500 µl of PBS to a PD-10 column. Intact ligand eluted in the void volume and degraded ligand in the total volume. Cell-associated ligand was quantified by solubilising the cell layer with 1 % SDS, followed by counting in a gamma counter.

Fluorescence microscopy of EECs following administration of F-LPS

Cultures of aEECs were established on glass coverslips as described above. The cultures were incubated with F-LPS (10–50 µg ml^{-1}) for 2 h at 12–14 °C and then fixed in 2.5 % glutaraldehyde and embedded in an antifading medium (Dako Fluorescent mounting medium, Glostrup, Denmark). The specific fluorescence due to F-LPS was observed as a bright green to yellow colour. Sections were examined using a Zeiss Axiophot photomicroscope equipped with incident-light fluorescence optics (Carl Zeiss, Obercochen, Germany). Pictures were taken on Kodak 64T or Ectachrome EPL 800 film (Kodak, Tokyo, Japan).

Results

Characterisation of native and modified LPS

Analysis by SDS–PAGE showed that the LPS derivative and F-LPS migrated the same distance as native LPS. Western blotting showed that binding of the monoclonal antibodies 7F3 and 2B5 to the LPS derivative and F-LPS was not reduced compared with native LPS (results not shown).

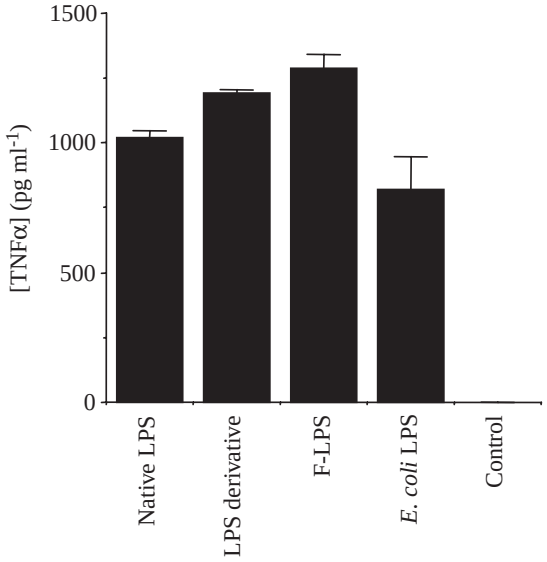

Fig. 1. *In vitro* release of tumour necrosis factor alpha (TNFα) from peritoneal macrophages in Balbc mice. TNFα was measured in the cell culture supernatants after 24 h of stimulation with lipopolysaccharide (LPS) (1 µg ml^{-1}). The control is supernatant from non-stimulated cultures. The results are presented as the mean + S.E.M. of three independent experiments. F-LPS, fluorescein-labelled LPS.

The biological activity of different preparations of LPS was measured as their ability to induce TNFα production by murine peritoneal macrophages. Monitoring of TNFα production revealed that modified *V. salmonicida* LPS and native LPS from either *V. salmonicida* or *E. coli* stimulated the murine peritoneal macrophages to produce similar levels of TNFα. No TNFα was produced in the absence of LPS (Fig. 1).

Anatomical distribution of [^{125}I]LPS

Trace amounts of [^{125}I]LPS were injected intravenously, and the amount of radioactivity in various tissues was recorded after 1 h, 4 h, 24 h and 48 h (Fig. 2). Approximately 41 % of the total radioactivity recovered was found in the heart 1 h after injection; 47 h later, the amount was still 41 %. The total amount of radioactivity in the blood decreased from approximately 21 % after 1 h to 10 % after 48 h, while the amount found in the liver increased from 9 % to 13 % over the same period. The amounts of radioactivity found in the spleen, anterior kidney and intestine were low. The total amount of radioactivity found in the carcass increased from 24 % after 1 h to 30 % after 48 h.

The specific radioactivity in heart increased from approximately 1838×10^3 cts min^{-1} g^{-1} tissue 1 h after intravenous injection to 2150×10^3 cts min^{-1} g^{-1} tissue 48 h after injection. The specific radioactivity in blood decreased from 44×10^3 cts min^{-1} g^{-1} tissue after 1 h to 15×10^3 cts min^{-1} g^{-1} tissue after 48 h. The corresponding figures were approximately 19×10^3 to 19×10^3 cts min^{-1} g^{-1} tissue for the spleen, 6.5×10^3 to 9.4×10^3 cts min^{-1} g^{-1} tissue for the liver, 39.4×10^3 to 32.6×10^3 cts min^{-1} g^{-1} tissue for the kidney and 4×10^3 to 8.8×10^3 cts min^{-1} g^{-1} tissue for the intestine. The specific radioactivity in the carcass was never higher than 3×10^3 cts min^{-1} g^{-1} tissue.

One hour after intravenous injection of trace amounts of [^{125}I]LPS, the content of radioactivity was highest in the atrium

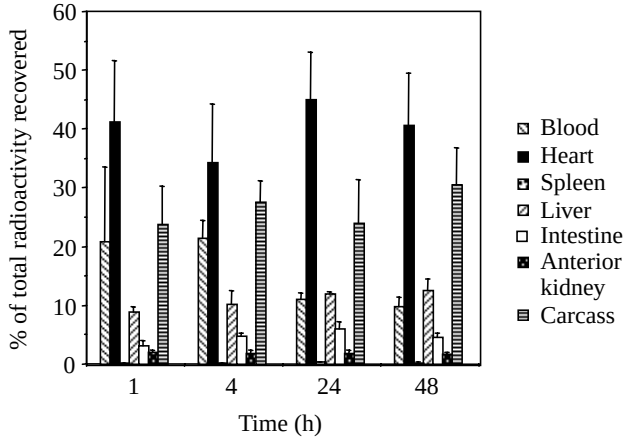

Fig. 2. Time course of the appearance of radioactivity in tissues of the Atlantic cod (*Gadus morhua* L.) after intravenous administration of trace amounts of ^{125}I-labelled lipopolysaccharide (0.5–1 µg kg^{-1} body mass). The results are expressed as means + S.E.M. of three fish per time point.

Table 1. *Ratio of tissue to blood specific radioactivity following intravenous injections of approximately 5 μg of ^{125}I-labelled lipopolysaccharide*

	[^{125}I]LPS	
	No inhibitor	+FSA
Atrium	16.78±9.27	1.10±0.32*
Ventricle	11.34±5.98	0.53±0.14*
Liver	0.17±0.08	0.11±0.03
Kidney	1.06±0.34	0.78±0.35
Spleen	0.84±0.65	0.45±0.12
Blood	1	1

Labelled ligands were injected alone or together with excess formaldehyde-treated serum albumin (FSA). The fish were killed 1 h after intravenous injection of ^{125}I-labelled lipopolysaccharide ([^{125}I]LPS).

Values are means ± S.E.M. (N=8). The ratio of tissue to blood specific radioactivity was calculated as (cts min^{-1} g^{-1} tissue)/(cts min^{-1} g^{-1} blood). The specific radioactivity in blood 1 h after injection of [^{125}I]LPS alone was $33.9\times10^3\pm18.9\times10^3$ cts min^{-1} g^{-1} tissue and with inhibitor $69.60\times10^3\pm35.96\times10^3$ cts min^{-1} g^{-1} tissue.

*Significantly different from the value in the absence of FSA (P<0.05).

and ventricle when expressed as cts min^{-1} g^{-1} tissue. The high atrium to blood and ventricle to blood ratios (16.8:1 and 11.3:1) compared with the much lower ratios in liver, anterior kidney and spleen (0.2:1, 1.1:1 and 0.8:1 respectively) (Table 1), indicate a very effective uptake mechanism for LPS in the heart. Injection of [^{125}I]LPS together with excess amounts of unlabelled FSA effectively blocked uptake of radiotracer by the heart. It should be noted that the tissue to blood ratio of radioactivity in other organs was not significantly influenced by co-injection with FSA, indicating that FSA and LPS compete for the same specific receptor in heart tissue only.

Uptake of [^{125}I]LPS by blood cells was investigated by density gradient sedimentation of whole blood sampled 1 and 24 h after injection of [^{125}I]LPS. After 1 and 24 h, only 5 % of the total amount of blood radioactivity was found in the fractions containing fish leukocytes. The fractions with red blood cells contained 2 % of the radioactivity after 1 h, increasing to 4 % after 24 h. The largest amounts of radioactivity (>90 %) were found in the fractions containing plasma, both 1 and 24 h after injection. The amount of radioactivity in whole blood decreased from 13.8×10^3 cts min^{-1} ml^{-1} blood after 1 h to 6.1×10^3 cts min^{-1} ml^{-1} blood after 24 h.

Cellular distribution of native LPS

The use of monoclonal anti-native-LPS antibodies to stain tissue sections prepared from fish that had been injected with native LPS revealed the presence of LPS in endocardial cells in both the atrium (Fig. 3A) and the ventricle (Fig. 3B) 48 h after injection. LPS was also detected in cells of the interstitium of the anterior kidney (Fig. 3C) and spleen (results not shown) 48 h after administration.

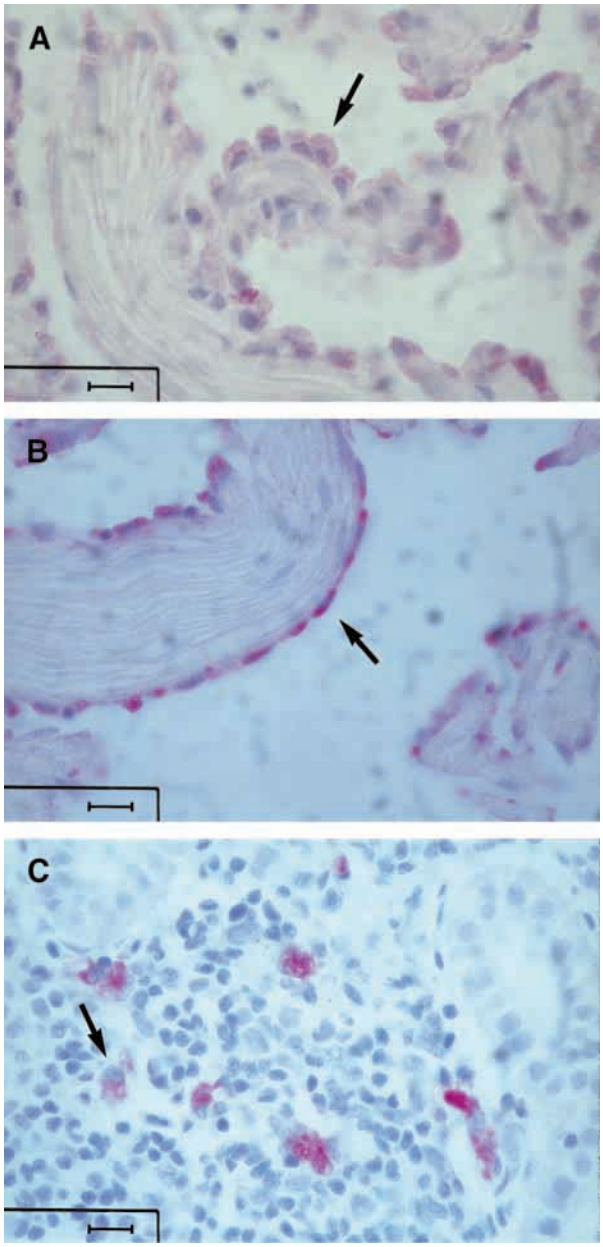

Fig. 3. Immunohistochemistry on (A) a section of atrium, (B) a section of ventricle and (C) a section of anterior kidney 48 h after intravenous administration of unlabelled lipopolysaccharide. Reaction product (arrows) was observed within endocardial endothelial cells (A,B) and probably in macrophages (C). Scale bars, 10 μm.

Endocytosis LPS in cod aEECs and rat sLECs in vitro

F-LPS was incubated for 1 h with cultured aEECs. Subsequent fixation of the cultures and examination in the fluorescence microscope revealed that all cells accumulated large amounts of fluorescent material in distinct vesicles (Fig. 4).

A series of experiments was then performed to study the specificity and kinetics of endocytosis of [^{125}I]LPS in cultured aEECs. Since endocytosis of LPS has been studied previously

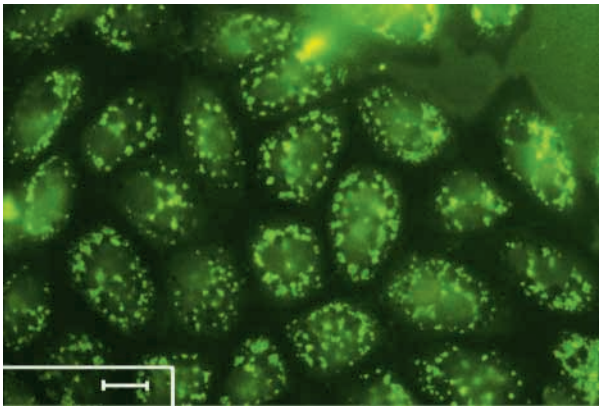

Fig. 4. Fluorescence micrograph of atrial endocardial endothelial cells cultured on a glass coverslip (pooled cells from three fish) and incubated with fluorescein-labelled lipopolysaccharide for 1 h at 12 °C. All endocardial cells in the monolayer cultures accumulated the probe. Note that fluorescence is confined to discrete vesicles probably representing endocytic vesicles. Scale bar, 10 μm.

in sLECs (Shnyra and Lindberg, 1995), which have been reported to play the same physiological role in mammals as EECs do in cod (Smedsrød et al., 1995), we wanted to compare the specificity of uptake of [^{125}I]LPS in aEECs with that in sLECs. In addition, since uptake of LPS by sLECs has been reported to be mediated *via* the scavenger receptor (Shnyra and Lindberg, 1995), we tested a selection of known ligands for the sLEC scavenger receptor to determine whether they would also serve as competitive inhibitors for the uptake of [^{125}I]LPS by aEECs. F-LPS, FSA, fucoidan and dextran sulphate, each used at a concentration of 100 μg ml^{-1}, inhibited endocytosis of [^{125}I]LPS in aEECs by 80 % (Fig. 5). In rat sLECs, F-LPS

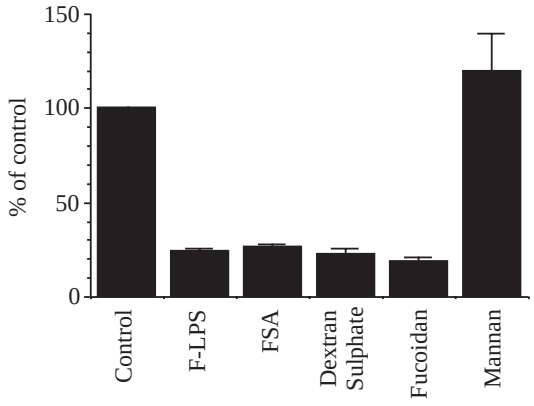

Fig. 5. Specificity of endocytosis of ^{125}I-labelled lipopolysaccharide in cultured cod atrial endocardial endothelial cells (aEECs). Monolayer cultures of aEECs in 2 cm^2 wells were incubated for 2 h at 12 °C with trace amounts of labelled ligand (approximately 2×10^4 cts min^{-1}; 3 ng) alone (Control) or together with excess amounts of unlabelled macromolecules (100 μg ml^{-1}). The results are presented as a percentage of the control value and are means + S.E.M. of three independent experiments. F-LPS, fluorescein-labelled lipopolysaccharide; FSA, formaldehyde-treated serum albumin.

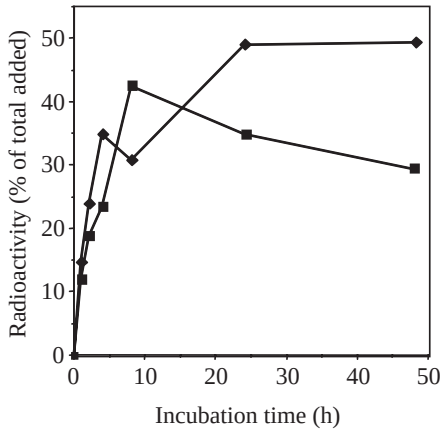

Fig. 6. Kinetics of endocytosis of ^{125}I-labelled lipopolysaccharide ([^{125}I]LPS) in cultured cod atrial endocardial cells (aEECs). Monolayer cultures of aEECs in 2 cm^2 wells were incubated with trace amounts of [^{125}I]LPS (approximately 2×10^4 cts min^{-1}; 3 ng) at 12 °C. The results are presented as the cell-associated percentage of the total added radioactivity and are the results from two independent experiments.

and FSA inhibited endocytosis of [^{125}I]LPS by 80 % (data not shown). Mannan (100 μg ml^{-1}), a ligand for the mannose receptor, had no inhibitory effect on the endocytosis of [^{125}I]LPS in either aEECs (Fig. 5) or sLECs (data not shown). F-LPS inhibited endocytosis of [^{125}I]FSA in cultured cod aEECs by more than 90 % (data not shown).

To study the kinetics of endocytosis, cultured aEECs were incubated with trace amounts of [^{125}I]LPS for 1, 2, 4, 8, 24 and 48 h (Fig. 6). Approximately 13 % of the added ligand was found as cell-associated radioactivity after 1 h. The cell-associated radioactivity increased to 41 % after 24 h, and this level did not increase with further incubation. The proportion of radioactivity found as low-molecular-mass material in cell culture supernatants was negligible, even after 48 h of culture (results not shown). Similarly, chromatography of solubilised cells after 48 h of incubation with trace amounts of [^{125}I]LPS demonstrated no intracellular degradation of the endocytosed LPS (Fig. 7). Crystal Violet staining of nuclei demonstrated that prolonged incubation of aEECs with 10 μg ml^{-1} LPS did not reduce viability (results not shown).

Discussion

We have investigated the tissue distribution and cellular localisation of R-type *V. salmonicida* LPS in Atlantic cod. Non-derivatised LPS, or LPS tagged with fluorescence or radioactivity, was administered intravenously to cod or incubated with cultured cod aEECs *in vitro*. The specificity of uptake was examined *in vivo* and *in vitro* by competitive ligand inhibition. For the *in vivo* and *in vitro* receptor specificity studies, LPS was labelled with ^{125}I according to Ulevitch (1978). The resulting specific radioactivity of [^{125}I]LPS was sufficiently high to allow studies of binding of LPS to cultured aEECs. The chemical treatments required for radioiodination

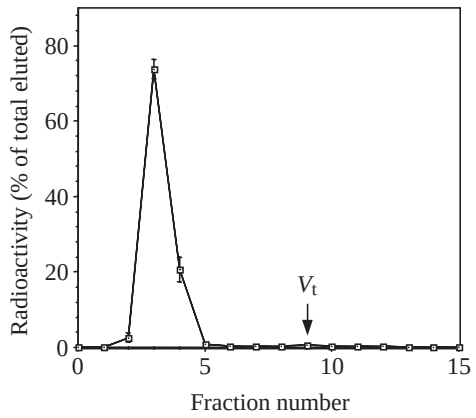

Fig. 7. Intracellular degradation of endocytosed ^{125}I-labelled lipopolysaccharide ([^{125}I]LPS). Monolayer cultures of cod atrial endocardial cells in 2 cm^2 wells were incubated for 48 h with [^{125}I]LPS (approximately 2×10^4 cts min^{-1}; 3 ng). Solubilised cultures were analysed by gel filtration on a PD-10 column. The ordinate indicates the percentage of total eluted radioactivity. The results are mean of six wells ± S.E.M. V_t, total volume.

did not alter the immunological or biological properties of *V. salmonicida* LPS. This finding is in accordance with the results of Ulevitch (1978).

The present results reveal that the uptake of trace amounts (micrograms) of R-type *V. salmonicida* [^{125}I]LPS after intravenous administration is greatest in the cod heart, where approximately 40 % of the total recovered radioactivity was found. The specific radioactivity in the heart tissue was 2150×10^3 cts min^{-1} g^{-1} tissue after 48 h. The amounts of radioactivity found in spleen, liver, head kidney and intestine were lower compared with the amounts found in the heart. The total amount of radioactivity found in the carcass was high, but the specific radioactivity was less than 3×10^3 cts min^{-1} g^{-1} tissue. Dalmo et al. (1998) investigated the specific uptake of tritiated S-type LPS after intravenous injection of a relatively high dose (milligrams) in Atlantic cod. They found a high specific radioactivity in the heart, spleen and kidney, but they did not report the total amount of radioactivity recovered from these organs.

Distribution studies performed in rabbit and rat have shown that circulating LPS is taken up mainly in the liver (Mathison and Ulevitch, 1979). The Kupffer cells of these mammals are largely responsible for the clearance, while sLECs and parenchymal cells contribute to a lesser extent (Ruiter et al., 1981; van Oosten et al., 1998). Kupffer cells constitute 80–90 % of the total fixed macrophages in the mammalian body (Phillips, 1989). Unlike mammals, most fish that have been investigated seem to lack a resident sinusoidal macrophage population in the liver (Speilberg et al., 1994). The anterior kidney of Atlantic cod and other teleosts is a haematopoietic organ and a rich source of macrophages (Braun-Nesje et al., 1981; Sørensen et al., 1997). The fact that only 1.7 % of the total radioactivity was recovered from the anterior kidney of cod 48 h after injection of trace amounts of [^{125}I]LPS suggests that cod macrophages, in contrast to mammalian macrophages, play a minor role in the clearance of [^{125}I]LPS. Analysis of blood 24 h after injection of [^{125}I]LPS demonstrated that the blood fraction containing leukocytes was associated with only a minor amount of the total radioactivity found in blood. The level of radioactivity was fairly stable over the experimental period (24 h), indicating that migration and recruitment of leukocytes containing LPS from other organs/sites into the general circulation was unlikely to have occurred.

When trace amounts of [^{125}I]LPS were co-injected with excess amounts of unlabelled FSA, the tissue to blood ratios measured after 1 h in the atrium and ventricle were reduced by more than 95 %, indicating that uptake of LPS in the heart is largely mediated *via* the scavenger receptor. The amount of radioactivity in all the other internal organs was lower than that in the blood in all experiments. The cod heart is the most important scavenger organ for circulating FSA and amino-terminal propeptides of type I procollagen (PINP), both of which represent ligands for the scavenger receptor (Sørensen et al., 1998). Sørensen et al. observed an increase in liver-associated radioactivity when trace amounts of [^{125}I]FSA were co-injected with excess amounts of unlabelled FSA or F-FSA. On the basis of this finding, they suggested an alternative route of uptake of FSA in cod liver. They also suggested that the liver receptor, in comparison with the heart receptor, may have a lower affinity, but larger capacity, for scavenger receptor ligands. In contrast, our results indicate that the [^{125}I]LPS that is not cleared by the heart is not cleared by the liver since the tissue to blood ratio in liver is less than 1 over the entire experimental period.

The cellular localisation of LPS was investigated by injection of native LPS and subsequent detection by immunohistochemistry. Immunostaining could be seen in EECs of the atrium and ventricle. Immunostaining was also detected in macrophage-like cells in the anterior kidney, albeit to a much lesser extent than in heart. Part of this uptake may reflect phagocytosis of LPS aggregates or uptake of complement-C3-bound LPS by macrophages since LPS is a known activator of the fish complement pathways (Boesen et al., 1999). In addition, the rather high dose of LPS injected in these experiments to ensure immunohistochemically detectable levels would certainly have saturated the uptake mechanism in the EECs, causing significant amounts of LPS to circulate for an extended period. Opportunistic and less specific uptake mechanisms in anterior kidney cells may therefore come into action. The cellular localisation of R-type LPS observed in the present study corresponds to the distribution of *Aeromonas salmonicida* S-type F-LPS in cod reported by Dalmo et al. (1998).

In vitro studies performed in pure cultures of cod aEECs demonstrated that F-LPS was internalised under serum-free conditions. Fluorescence was observed in discrete vesicles in all aEECs following a 1 h incubation. These structures probably represent endocytic vesicles. The observation that the *in vitro* uptake of [^{125}I]LPS was effectively inhibited by excess

amounts of scavenger receptor ligands supports the idea that LPS is taken up *via* the scavenger receptor expressed by these cells. Sørensen et al. (1998) demonstrated that the uptake of the scavenger receptor ligands FSA and PINP was inhibited by co-incubation of excess amounts of FSA. Work done by Shnyra and Lindberg (1995) and van Oosten et al. (1998) in purified cultures of sLECs from the rat liver have demonstrated that these cells are able to bind and internalise *Salmonella minnesota* R595 lipopolysaccharide *in vitro*. Their results showed that polyanions were potent inhibitors of [^{125}I]LPS binding and that their inhibitory effect resulted from the interactions between these polymers and membrane LPS-binding sites. They suggested that a scavenger receptor was responsible for cellular binding of LPS to sLECs. In our hands, inhibition studies with *Vibrio salmonicida* [^{125}I]LPS in sLECs confirmed these results.

Studies in mammals have shown that CD14, the macrophage scavenger receptors and toll receptors on the surface of monocytes and macrophages bind the lipid A moiety of LPS (Aderem and Ulevitch, 2000). Toll receptors, at least, are capable of initiating signals to the cell, resulting in the activation of various functions such as phagocytosis, endocytosis and bactericidal defence (Aderem and Ulevitch, 2000). The mammalian scavenger receptor does not appear to function as a signalling receptor for LPS. Rather, it has been proposed that this receptor plays a protective role in host defence by scavenging potentially harmful substances such as LPS and, thereby, reducing the release of pro-inflammatory cytokines (Fenton and Golenbock, 1998). At present, it is not known whether fish possess LPS receptors apart from the scavenger receptors, and the absence of LPS receptors to mediate intracellular activation may explain their observed tolerance to endotoxin (Berczi et al., 1966).

The uptake of [^{125}I]LPS by aEECs *in vitro* was rapid, with approximately 13 % of the added [^{125}I]LPS being taken up after 1 h and 41 % by 24 h. Further incubation did not increase the amount of cell-associated radioactivity. The rate of uptake of [^{125}I]LPS during the first 24 h is similar to that of [^{125}I]PINP *via* the cod aEEC scavenger receptor, but slower than endocytosis of [^{125}I]FSA by the same receptor (Sørensen et al., 1998). The corresponding values for [^{125}I]FSA were 25 % after 1 h and 75 % after 6 h of incubation. The uptake of [^{125}I]PINP was slower than for [^{125}I]FSA (4 % after 1 h and 55 % after 24 h).

Recent studies in our laboratory showed that as much as 45 % of added [^{125}I]FSA and [^{125}I]PINP were degraded and recovered as low-molecular-mass products in the medium after 24 h of incubation with cultured aEECs (Sørensen et al., 1998). Against this background, it is interesting to note that we observed practically no degradation of [^{125}I]LPS, even after 48 h of incubation. Although deacetylation of the lipid A moiety of LPS has been observed in mammalian macrophages and granulocytes (Munford and Hall, 1985, 1986), complete enzymatic degradation of LPS in vertebrate cells is unlikely because of the absence of hydrolases with specificities for the microbial sugar and peptidoglycan linkages of LPS. This, together with reports that immunoreactive LPS can be detected immunohistochemically in mammalian and fish tissues several weeks after injection of LPS (Ge et al., 1994; Lamers and Pilarczyk, 1982), fits well with our observation that only minor amounts of [^{125}I]LPS was degraded in cultured aEECs even after 48 h of incubation.

The uptake of [^{125}I]LPS by cultured aEECs levelled off after 24 h, and no further uptake was observed over the next 24 h of incubation. We speculate that, if the aEECs lack the capability to degrade [^{125}I]LPS, this will cause a saturation of the endocytic capacity of the aEECs either by inhibiting the recirculation of receptor molecules, and thereby reducing the number of receptors on the cell surface, or by blocking other parts of the endocytic apparatus. Results from rat sLECs have demonstrated that endocytosis of advanced glycation end-products *via* the sLEC scavenger receptor results in a reduced ability to endocytose scavenger receptor ligands in general (Hansen et al., 2000). Further experiments are under way in our laboratory to investigate whether accumulation of LPS in aEECs may affect the general scavenger function of these cells. Nuclear staining and subsequent cell counting of aEECs incubated with 10 μg ml^{-1} LPS for 48 h showed that normal cell numbers were present in the wells. This indicates that LPS was not toxic for the cells and that the reduced uptake cannot be explained by cell death.

In conclusion, our results indicate that the population of EECs is capable of taking up LPS from the circulation of the Atlantic cod and that LPS is internalised in a scavenger-receptor-dependent manner both *in vivo* and in cultures of aEECs. Moreover, these cells, which degrade an array of endocytosed macromolecules, are unable to degrade LPS.

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