Available online at www.sciencedirect.com
SCIENCE DIRECT®
Vaccine 21 (2003) 4178–4193
Vaccine
www.elsevier.com/locate/vaccine

Increased efficacy of immersion vaccination in fish with hyperosmotic pretreatment

Mark O. Huising [a,b,*], Teun Guichelaar [a], Casper Hoek [a], B.M. Lidy Verburg-van Kemenade [a], Gert Flik [b,1], Huub F.J. Savelkoul [a], Jan H.W.M. Rombout [a]

[a] *Department of Cell Biology and Immunology, Wageningen University, Marijkeweg 40, P.O. Box 338, 6700 AH Wageningen, The Netherlands*
[b] *Department of Animal Physiology, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands*

Received 10 December 2002; received in revised form 22 April 2003; accepted 18 June 2003

Abstract

Immersion vaccination is common practice in aquaculture, because of its convenience for mass vaccination with sufficient protection. However, the mechanisms of antigen uptake and presentation, resulting in a protective immune response and the role of the innate immune system therein are largely unknown. The impact of immersion vaccination on fish physiology and on the ensuing innate and specific immune response was characterized with fluorescently labeled particulate and soluble model antigens. Vaccination of common carp by direct immersion (DI) or hyperosmotic immersion (HI; direct immersion, preceded by a brief immersion in a hypertonic solution) greatly enhanced the uptake of soluble, but not particulate antigen through temporary disruption of the integrity of the epithelia of gills and skin. Damage induced is mild and does not impose additional stress over the handling associated with immersion vaccination. Especially HI briefly but strongly activates the innate immune system. We conclude that HI more effectively increased the uptake of vaccine and enhanced the efficacy by which vaccine components are processed and presented by the innate immune system, dually enhancing the mucosal immune response. Understanding the mechanisms involved in uptake and processing of vaccine in the early phase of the immune response will greatly benefit the design of immersion vaccination.

Keywords: Hyperosmotic immersion; Bath vaccination; *Aeromonas salmonicida*

1. Introduction

During the last 15 years fish aquaculture has more than doubled [1]. The need for protection from infectious disease in high-density fish farming conditions has increased in parallel. Fish vaccines are commonly administered through one of three possible routes. First, intraperitoneal (i.p.) injection is widely used, especially in salmonid fish farming, but injection is labor intensive and costly, and in combination with viscous oil-based adjuvants only feasible on a commercial scale in salmonids and only from the smolt stage onwards [2]. Second, immersion vaccination is an established practice in aquaculture, e.g. with commercial vaccines for pathogens such as *Vibrio* spp., but not for others [3]. Immersion vaccination is a particularly cost effective method of administration in very small fish. However, immune responses following immersion vaccination are generally less robust and of shorter duration as those obtained through i.p. injection [4]. Third, oral vaccination is an option but still largely in an experimental stage. Research in this field focuses on protection of the vaccine from digestion in the early digestive system through encapsulation [5,6]. Besides the route of vaccine administration other factors such as the nature of the antigen, the use of adjuvants, vaccine dose (and in case of immersion vaccination vaccine exposure time), developmental stage, immune and nutritional status and temperature can have great impact on the overall efficacy of the resulting immune response [4,7–11].

Although vaccination through i.p. injection tends to generate immune responses that are more robust and last longer, immersion vaccination oftentimes is the method of choice. An additional advantage of immersion is that vaccine delivery is through the same route as that utilized by many fish pathogens, generating topologically specific mucosal immunity, i.e. where the encounter with a pathogen is most likely [12–15].

Vaccine delivery in immersion vaccination may vary. Most commonly used are direct immersion (DI), hyper-

* Corresponding author. Tel.: +31-317-483965; fax: +31-317-483955.
E-mail address: mark.huising@wur.nl (M.O. Huising).
[1] Fax: +31-24-3653229.

0264-410X/$ – see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0264-410X(03)00497-3

osmotic immersion (HI) and spray vaccination, the latter mostly applied to larger individuals. In all cases vaccine uptake is thought to occur largely along the mucosal surfaces of the fish, i.e. the gills and skin [16–18]. Some authors have also implicated the lateral line [19] and the intestine [8,20] as sites of significant antigen uptake.

In the past the added value of hyperosmotic immersion over direct immersion is refuted. Most authors claim higher uptake of soluble antigen following HI [7,16,19]. However, the benefit of higher vaccine uptake is often discarded, as the stress and the damage to the integument are considered too great [4]. Substantial data corroborating this notion have to the best of our knowledge not been published. The uptake of particulate antigen is less affected by immersion in a hypertonic solution, considering that uptake [21,22] and immune responses [23] reported in HI and DI protocols are comparable. This is noteworthy because most commercial immersion vaccines are bacterins, consisting of formalin fixed bacteria and their soluble excretions. It remains to be assessed whether particulate, soluble, or both components of these vaccines contribute to eventual immunity.

The efficacy of immersion vaccination is mostly evaluated by antibody production and/or survival upon challenge. Immersion vaccination: (1) induces detectable systemic [23–26] and mucosal [12,15] antibody responses, (2) confers protection upon challenge [27,28] or (3) both [29,30]. However, some reports indicate a transient [31] humoral response or protection without a detectable antibody response [32], indicating that the exact mechanisms of protection are sometimes enigmatic.

We here characterize and compare the physiological and immune responses to immersion vaccination through HI and DI protocols in common carp (*Cyprinus carpio*). We employed an *Aeromonas salmonicida* bacterin, *A. salmonicida* lipo-polysaccharide (LPS) of the same bacterial strain and bovine serum albumin (BSA), all fluorescently labeled, as model antigens. This enabled us to analyze physiological and immune responses following immunization with particulate versus soluble antigen as well as compare uptake and immune response following immersion with soluble protein and LPS. The latter is of interest since it is conceivable that protective immunity is evoked by a combined response to both soluble moieties [33]. Subsequently, important components of the innate and acquired immune system as well as physiological and endocrine parameters were analyzed to conclude that HI protocols may provide cost-effective fish vaccination.

2. Materials and methods

2.1. Animals

Common carp (*Cyprinus carpio* L.) were reared at 23 °C in recirculating UV-treated tap water at the 'De Haar Vissen' facility in Wageningen. Fish were fed pelleted dry food (Provimi, Rotterdam, The Netherlands) at a daily rate of 0.7% of their estimated body weight. R3 × R8 are the hybrid offspring of a cross between fish of Hungarian origin (R8 strain) and fish of Polish origin (R3 strain) [34]. Fish used for analysis of humoral responses were vaccinated at 14 weeks post fertilization. Animals used for confocal laser scanning microscopy, flow cytometry and RQ-PCR were 10–24 weeks of age. Carp (12 weeks) of the same strain housed under identical conditions at the fish facilities of the Department of Animal Physiology at the University of Nijmegen were used for determination of serum osmolality, cortisol and ion concentrations and electron microscopy. Animals in one experiment were always reared from the same offspring.

2.2. Blood collection and tissue preparation

Fish were anaesthetized with 0.2 g l^{-1} tricaine methane sulphonate (TMS) buffered with 0.4 g l^{-1} $NaHCO_3$ or with 0.1% 2-phenoxyethanol. Blood was obtained by puncture of the caudal vessel using a heparinized (Leo Pharmaceutical Products Ltd., Weesp, The Netherlands) syringe fitted with a 21 or 25 Gauge needle and processed for further analysis according to the requirements of the various techniques used. Gills were isolated by carefully excising whole gill arches. Skin samples were obtained by taking a transverse slice of ca. 0.5 cm out of the base of the tail. Anterior kidney was surgically removed.

2.3. Fluorescein conjugation

A bacterin (20 ml) containing formalin-inactivated *A. salmonicida* (MT004; [35] at 1.2×10^8 bacteria ml^{-1} was adjusted to pH 9.0 with 0.5 M $NaHCO_3$ (pH 9.5). Subsequently, 4 ml 2.5 mg ml^{-1} fluorescein 5-isothiocyanate (FITC; F-7250, Sigma) in dimethylsulfoxide (DMSO) was added and the reaction mixture was stirred gently at room temperature in the dark for 4 h. Free FITC was removed by extensive dialysis (Spectra/Por dialysis tube, MWCO 6–8.000 Da) in 4 l 0.1× phosphate buffered saline without magnesium salts (PBS^-; 0.8 g l^{-1} NaCl, 0.02 g l^{-1} KCl, 0.02 g l^{-1} KH_2PO_4, 0.144 g l^{-1} Na_2HPO_4, pH 7.40). Bacterin fluorescence was microscopically confirmed. The bacterin was filled to a final volume of 100 ml with 0.1× PBS^- resulting in 2.4×10^7 bacteria ml^{-1} and stored at 4 °C until use. *A. salmonicida* (MT004) crude lipo-polysaccharide (>80% pure, lyophilized; gift from Dr. I.R. Bricknell, Aberdeen) was prepared as a 2% (w/v) solution in 10 ml 0.5 M $NaHCO_3$ (pH 9.5). 5-([4,6-Dichlorotriazin-2-yl]amino)fluorescein (DTAF); D-0531, Sigma) was used for conjugation to LPS, since it reacts directly with polysaccharides at room temperature at pH above 9.0 [36]. DTAF was dissolved in DMSO (50 mg ml^{-1}) and added. The reaction mixture was stirred gently at room temperature in the dark for 4 h. Free DTAF was removed by extensive

dialysis (Spectra/Por dialysis tube, MWCO 6–8.000 Da) in 4 l 0.1× PBS^- and the solution was filled to a final volume of 100 ml with 0.1× PBS^- resulting in 0.2% (w/v) LPS, filter sterilized (0.22 μm, Millipore) and stored at 4 °C until use. Bovine serum albumin (Fraction V; Roche diagnostics) was prepared as a 5% (w/v) solution in 100 ml 0.1× PBS^-. The pH was adjusted to 9.0 with 0.5 M $NaHCO_3$ (pH 9.5) and 20 ml 2.5 mg ml^{-1} FITC in DMSO was added. The reaction mixture was stirred gently at room temperature in the dark for 4 h. Free FITC was removed by extensive dialysis in 4 l 0.1× PBS^-. BSA-FITC was precipitated overnight at 4 °C by adding 62 g $(NH_4)_2SO_4$ and spun down 20 min at 12,000 × *g*. Pellet was washed with saturated $(NH_4)_2SO_4$ and dissolved in 100 ml 0.1× PBS^-. This solution was dialyzed 10× to 4 l 0.1× PBS^-, filled to a total volume of 250 ml with 0.1× PBS^-, filter sterilized (0.22 μm, Millipore) and stored at 4 °C until use. All conjugates were made fresh for each experiment. Absence of fluorescence from the spend dialysis buffer was confirmed spectrophotometrically at 495 nm. The DTAF/LPS ratio in the final mixture was determined to be 2.0–2.3 and the FITC/BSA ratio was determined to be 4.0–5.0 according to the following formula: $[2.87 \times abs_{495\,nm}/(abs_{280\,nm} - 0.35 \times abs_{495\,nm})]$ [37].

2.4. Immersion methods

Fish subjected to HI were immersed in 4.5% (w/v) NaCl (1450 mOsm kg^{-1}; aerated overnight before use) for 2 min and immediately net transferred to vaccine solution for 10 min. Fish subjected to DI were immersed in vaccine solution for 10 min. LPS–DTAF (0.2% (w/v)), *A. salmonicida* bacterin-FITC (2.4×10^7 bacteria ml^{-1}) or BSA-FITC (2% (w/v)) were used as vaccine solutions. The high salinity of the hyperosmotic solution caused the fish to passively float to the surface. After vaccination fish were returned to their tanks. For determination of plasma parameters, fish were only subjected to (sham) hyperosmotic immersion and not exposed to the actual vaccine.

2.5. Electron microscopy

Gill and skin tissue was prefixed on ice in 3% glutaraldehyde (EM grade) in 0.1 M Na-cacodylate buffer 15 min and fixed on ice in 1% OsO4 (prefix buffer:2% OsO4:distilled water; 1:2:1) for 1 h. Samples were washed three times in distilled water and stored at 4 °C. For transmission electron microscopy fixed tissue was embedded in Epon and examined using a Jeol 100 CX transmission electron microscope. For scanning electron microscopy samples were dehydrated (ethanol 30, 50, 70, 80 and 90%; 2× 100%; dried methanol 100%) critical-point-dried using liquid CO_2, mounted on a sample holder, covered with gold and examined using a Jeol JSM-T300 scanning electron microscope. Two individuals per treatment were extensively examined by SEM and TEM.

2.6. Confocal microscopy

Gill and skin tissue was fixed overnight in 4% paraformaldehyde in PBS^-. Gill filaments were carefully removed from the gill arch using micro-instrumentation and embedded in Vectashield containing propidium iodide (PI; Vector laboratories Inc.). Transversal skin sections of 100 μm thick were sliced by a vibratome (Vibratome 1500 Sectioning System) and embedded in Vectashield containing PI. Gill and skin samples of two individuals per treatment were extensively examined with a Zeiss LSM-510 laser scanning microscope. Photographs of samples of the same magnification were always taken using identical microscope settings at those settings that yielded only minimal background in the green spectrum in the negative controls. Fluorescein signal was excited using a 488 nm argon laser and detected using a band-pass filter (505–550 nm) and PI signal was excited using a 543 helium–neon laser and detected with a long-pass filter (585 nm).

2.7. Cortisol RIA

Freshly collected heparinized blood was spun down in a microcentrifuge (10 min at 9300 × *g*) at 4 °C. Plasma was taken off and stored at −20 °C until use. Cortisol was measured by radioimmunoassay [38], using a commercial antiserum (Bioclinical Services Ltd., Cardiff, UK). All constituents were in phosphate-EDTA buffer (0.05 M Na_2HPO_4, 0.01 M Na_2EDTA, 0.003 M NaN_3, pH 7.4). Ten microliters of samples or standards in RIA buffer (phosphate-EDTA buffer containing 0.1% 8-anilio-1-napthalene sulfonic acid and 0.1% (w/v) bovine γ-globulin) were incubated with 100 μl antiserum (in RIA buffer containing 0.2% normal rabbit serum) for 4 h. Samples were incubated overnight with 100 μl iodinated cortisol (ca. 1700 cpm per tube; ^{125}I-cortisol, Amersham, Nederland BV, Hertogenbosch, The Netherlands) and 100 μl goat anti-rabbit γ-globulin (in RIA buffer). Bound and free cortisol were separated by adding 1 ml of ice-cold precipitation buffer (phosphate-EDTA buffer containing 2% (w/v) bovine serum albumin and 5% (w/v) polyethylene glycol). The tubes were centrifuged at 4 °C (20 min at 2000 × *g*), the supernatant aspirated and counted in a gamma counter (1272 clinigamma, LKB Wallac, Turku, Finland).

2.8. Plasma osmolality and ion concentrations

Blood was spun down fresh in a microcentrifuge (10 min at 9300 × *g*) at 4 °C. Plasma was taken and stored at −20 °C until use. Plasma osmolality was determined using a cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany). Plasma sodium and chloride concentrations were determined by flame photometry and ferrothiocyanate-based colorimetric procedures, respectively using a Technicon AutoAnalyzer (Pulse Instrumentation, Saskatchewan, Canada).

2.9. Flow cytometry

Gill and anterior kidney tissue was passed through a 50 μm nylon mesh and washed once with carp RPMI (cRPMI; RPMI 1640, Gibco) adjusted to carp osmolality (270 mOsm kg^{-1}) with 10% distilled water and containing 0.01% NaN_3 and 10 IU ml^{-1} heparin (Leo Pharmaceutical products BV, Weesp, The Netherlands). Blood was mixed with an equal volume of cRPMI and centrifuged 10 min at 100 × *g* to remove the majority of erythrocytes. The supernatant containing peripheral blood leucocytes (PBL) and the gill and anterior kidney cell suspensions were layered on discontinuous Percoll (Amersham Pharmacia Biotech AB) gradient (1.020 and 1.083 g cm^{-3}). Following centrifugation (30 min at 800 × *g* with brake disengaged) cells at the 1.083 g cm^{-3} interface were collected and washed in incubation buffer (cRPMI containing 1.0% (w/v) BSA) to remove Percoll. The cell pellet was resuspended in 100 μl incubation buffer containing 1:50 diluted mouse monoclonal antibody specific for carp neutrophils and macrophages (TCL-BE8) [39] (gift from Prof. N. Okamoto, Tokyo) and incubated on ice for 10 min. Cells were washed and centrifuged 10 min at 800 × *g* and the cell pellet was resuspended in incubation buffer containing 1:100 diluted secondary antibody (goat anti-mouse Ig-phycoerythrin (RPE) ($F(ab')_2$), Dako A/S, Denmark). Cells were washed and centrifuged as above. Controls incubated without antibody and with secondary antibody only were included for all samples. Cell suspensions were measured with a Beckman Coulter Epics XL-MCL using an excitation wave length of 488 nm and with the discriminator set at 100 in the FSC channel. FSC/SSC characteristics of 10,000 events were acquired in linear mode, fluorescence intensity at wave lengths of 525 (±10) and 575 (±10) nm was acquired at a log scale. Samples were analyzed using Expo32 software (Applied Cytometry Systems).

2.10. RNA isolation

RNA isolation was conducted according to Chomczynski and Sacchi [40]. Briefly, organs were homogenized in lysis buffer (4 M guanidium thiocyanate; 25 mM sodium citrate, pH 7.0; 0.5% sarcosyl; 0.1 M 2β-mercaptho-ethanol), followed by phenol/chloroform extractions. Total RNA was precipitated in ethanol, washed and dissolved in water. Concentrations were measured by spectrophotometry and integrity was ensured by analysis on a 1.5% agarose gel. RNA was stored at −80 °C for future use.

2.11. DNAse treatment and first strand cDNA synthesis

For each sample a non-template control was included. One microliter of 10× DNAse I reaction buffer and 1 μl DNAse I (Invitrogen, 18068-015) was added to 2 μg total RNA and incubated at room temperature, 15 min in a total volume of 10 μl. DNAse I was inactivated by adding 1 μl 25 mM EDTA and incubation at 65 °C, 10 min. To each sample 300 ng random hexamers, 1 μl 10 mM dNTP mix, 4 μl 5× First Strand buffer, 2 μl 0.1 M DTT and 10 U RNAse inhibitor (Invitrogen, 15518-012) were added and the mixture was incubated 10 min at room temperature and an additional 2 min at 37 °C. To each positive sample (but not the NT controls) 200 U Superscript RNAse H^- reverse transcriptase (RT; Invitrogen, 18053-017) was added and reactions were incubated 50 min at 37 °C. All reactions were filled up with demineralized water to a total volume of 1 ml and stored at −20 °C until further use.

2.12. Real-time quantitative PCR

Primer Express software (Applied Biosystems) was used to design primers for use in real-time quantitative PCR (RQ-PCR; Table 1). For tumor necrosis factor-α (TNFα) two slightly different reverse primers were designed and mixed in equimolar amounts to enable simultaneous detection of both isoforms [41]. Primers for α_2-macroglobulin (α_2M) were designed to detect all three known isoforms. For RQ-PCR 5 μl cDNA and forward and reverse primer (300 nM each) were added to 12.5 μl Sybr Green PCR Master Mix (Applied Biosystems) and filled up with demineralized water to a volume of 25 μl. RQ-PCR (2 min 48 °C, 10 min 95 °C, 40 cycles of 15 s 95 °C, and 1 min 60 °C) was carried out on a GeneAmp 5700 Sequence Detection System (Applied Biosystems). Data were analyzed using the ΔΔCt method [42] and the relative quantitation value expressed as $2^{-\Delta\Delta Ct}$.

2.13. Biosensor determination of antibody levels

Blood was spun down fresh in a microcentrifuge (10 min at 9300 × *g*) at 4 °C. Plasma was taken off and stored at −20 °C until use. Skin mucus was obtained by gently scraping the body surface of the anaesthetized fish with the blunt end of a scalpel and transferring the mucus to 50 μl 0.1× PBS^- containing 0.1% phenylmethanesulfonyl fluoride (PMSF) and 0.05% NaN_3. After thorough mixing the solid phase was spun down and the supernatant transferred to a new tube and stored at −20 °C until analysis. Plasma and mucus antibody levels were determined using a resonant mirror-based optical biosensor (IAsys plus, Affinity Sensors, Cambridge, UK) and a carboxymethyl-dextran cuvette coated with LPS–DTAF according to the manufacturers instructions. Data were analyzed using IAsys plus software (version 4.0.1, Affinity Sensors).

2.14. Statistics

All statistical analyses were carried out using SPSS software (version 10.1.0). Data were tested for normal distribution using the Shapiro–Wilk test. Homogeneity of variances was tested with the Levene test. For plasma cortisol, statistical analysis was carried out on the square root of the cortisol

Table 1
Primer sequences and corresponding EMBL acc. numbers

Gene	EMBL acc. numbers	Primer	Sequence
IL-1β	CCA245635	qIL-1b.fw1	CTGGAGCAATGCAATACAAAGTTC
		qIL-1b.rv1	CAAGGTAGAGGTTGCTGTTGGAA
TNFα1/TNFα2	AJ311800, AJ311801	qTNFa.fw1	GCTGTCTGCTTCACGCTCAA
		qTNFa.rv1a	CCTTGGAAGTGACATTTGCTTTT
		qTNFa.rv1b	GCCTTGGAAGTGACATTTTCTTTT
iNOS	CCA242906	qiNOS.fw1	CCCATGCAGTGGATGATAGGT
		qiNOS.rv1	TTAAACTCCTTGCATGCATCCTTA
α_2M	AB026128, AB026129, AB026130	qA2M.fw1	GACTGCCTTTGTCCTGAGGTCTT
		qA2M.rv1	TGGATAAAACAGCCGTCTGAATC
SAA	AB016524	qSAA.fw1	CAAGCCATTGGAGGTGCAA
		qSAA.rv1	TTCCCACGTGCATGGAAATA
β-actin	CCACTBA	qACT.fw1	CAACAGGGAAAAGATGACACAGATC
		qACT.rv1	GGGACAGCACAGCCTGGAT
40S ribosomal protein S11	AB012087	q40S.fw1	CCGTGGGTGACATCGTTACA
		q40S.rv1	TCAGGACATTGAACCTCACTGTCT

concentrations. Differences were evaluated using one-sided one factor analysis of variance (ANOVA). If ANOVA was significant, Dunnett's *t* test was used to determine which means differed significantly from the control. In case of non-homogeneous variances Dunnett's *C* test was used as a substitute for Dunnett's *t* test. Kruskal–Wallis *H* test was applied in case of non-normal distribution. If Kruskal–Wallis was significant, the Mann–Whitney *U* test was used to determine which means differed significantly.

3. Results

3.1. Plasma homeostasis

To study the impact of HI on homeostasis we analyzed the kinetics of plasma ion concentrations and osmolality following HI. Immediately after HI in 4.5% (w/v) NaCl, plasma levels of Na^+ (from 147 to 192 mM) and Cl^- (from 120 to 171 mM) as well as plasma osmolality (from 263 to 344 mOsm kg^{-1}) rose profoundly (Fig. 1). These levels returned to basal levels within 20 min, but at 3 h plasma Na^+ and Cl^- levels were slightly and transiently elevated once more to 169 and 144 mM, respectively.

3.2. Skin and gill morphology

The condition of the external epithelia was studied to ascertain whether changes of plasma parameters coincided with alterations in morphology. The outer layer of the skin epithelium of carp consisted of epithelial cells with a characteristic pattern of concentric microridges on the surface. In-between mucus-filled crypts of mucus cells were positioned at regular intervals (Fig. 2A). Transverse sections showed a multi-layered skin epidermis, with mucus cells and lymphocyte-like cells (Fig. 2B) as well as chloride cells and alarm cells dispersed between the outer epithelial layer and the basal membrane.

Immediately following HI, the majority of skin epithelial cells was still intact, but a considerable number (estimated 5%) of cells scattered throughout the epithelial surface was progressively losing their microridged surface, rounding up and shedding, leaving cell-size holes in the epithelium (Fig. 2C and D). On transverse TEM sections, the cytoplasm of these cells was less electron-dense, and the nuclei were losing their integrity (Fig. 2E). Twenty minutes after HI the holes in the epithelium were mostly sealed, but the microridges were very shallow and could have a villus-like rather than a ridge-like appearance (not shown). Three hours after HI the epithelium appeared normal and only differed from the control in their somewhat shallower microridges (Fig. 2F and G). Twenty minutes after DI, overall skin morphology was equal to untreated controls (not shown).

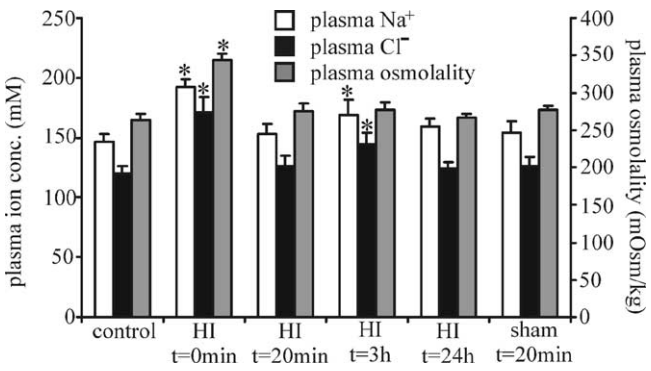

Fig. 1. Kinetics of plasma Na^+ and Cl^- concentrations and plasma osmolality at various times after the end of HI and sham immersion. Error bars denote S.D. of 10 replicate measurements. Asterisk indicates a significant difference from the control ($P < 0.001$).

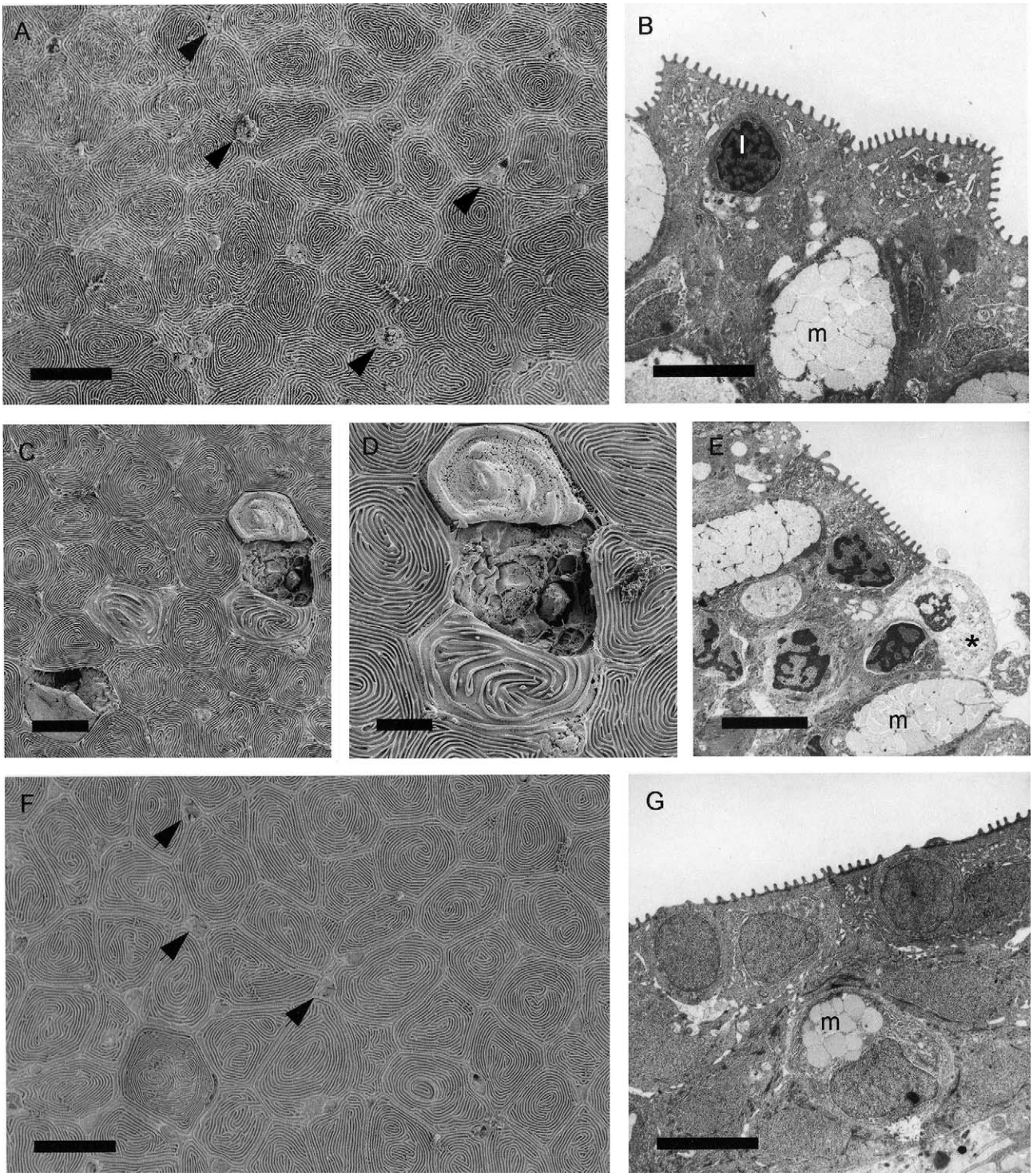

Fig. 2. Scanning and transmission electron micrographs of carp skin. Untreated carp (A and B) showed an intact continuous epithelium. Immediately after a HI (C–E) the majority of skin epithelial cells was still intact, but a considerable number of cells scattered throughout the epithelial surface was in various stages of losing their microridged surface, rounding up and shedding, leaving cell-size holes in the epithelium. Three hours after HI (F and G) the epithelial surface had recovered completely, with the exception of the more shallow microridges at the surface of the epithelium. m, mucus cell; l, lymphocyte. Arrowheads indicate crypts of mucus cells. Asterisk marks a damaged epithelial cell. Scale bars: (A, C and F) 10 μm; (B, D, E and G) 5 μm.

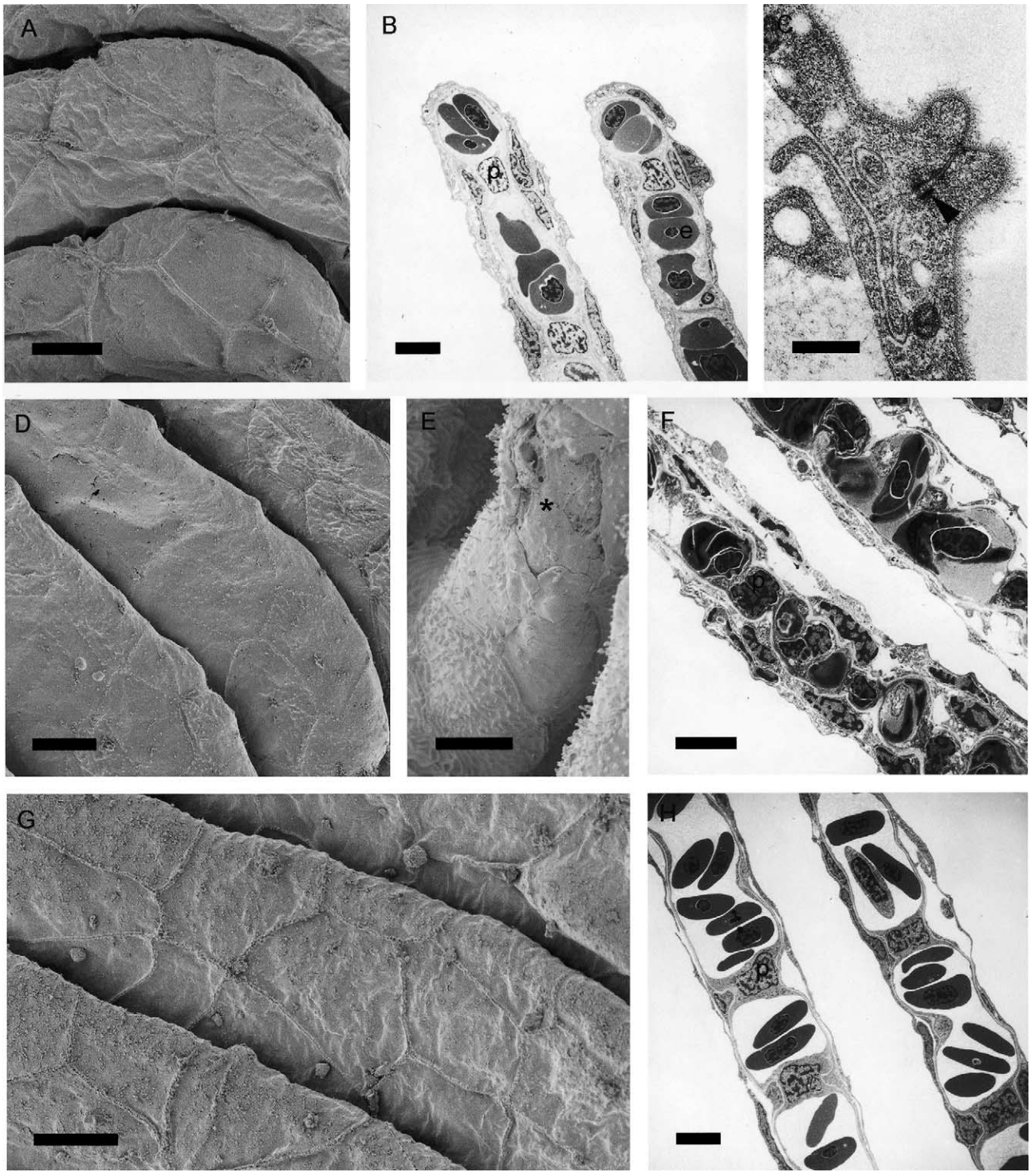

Fig. 3. Scanning and transmission electron micrographs of carp gills. Untreated carp (A–C) showed the intact external and internal organization of the lamellae. Immediately after HI (D–F) the pavement cells were mostly present, but the tight cell–cell contacts were not very prominent. At the base of the lamellae, some cells were shed, leaving holes in the epithelial surface. Three hours after HI (G and H) the gill surface had completely recovered. p, pillar cell; e, erythrocyte; The arrowhead indicates the tight junction between two pavement cells. Asterisk marks the cavity where a cell was shed. Scale bars: (A, D and G) 10 μm; (B, E, F and H) 5 μm; (C) 500 nm.

The lamellae of the gills in carp were covered by a very thin double layered epithelium consisting of smooth pavement epithelium cells (Fig. 3A). On transverse sections the internal anatomy of the gill lamellae was visible, consisting of a capillary bed bridged by pillar cells (Fig. 3B). The contacts between the pavement epithelial cells were raised, due to the presence of tight junctions. On the basal side of the epithelium the two cells form finger-like protrusions which were intricately entangled (Fig. 3C), ensuring a tight seal from the external milieu.

Directly after HI, the pavement epithelial cells were swollen, making the raised cell–cell contacts less prominent (Fig. 3D). Unlike the skin epithelium, few cells had shed and if so, they were located near the base of the lamellae (Fig. 3E), where the pavement cells began to develop villus-like protrusions in transition to the epithelium of the gill filament, with its characteristic microridges. Changes in the appearance of the filament epithelium were much alike those described above for skin epithelium. On transverse sections the internal anatomy of the lamellae was clearly disturbed, with substantial epithelial lifting (Fig. 3F). Twenty minutes after HI the pavement cells appeared normal again (not shown) and after 3 h the lamellar (Fig. 3G) as well as the filamental (not shown) epithelium was indistinguishable from that of controls. Lamellar anatomy had also recovered (Fig. 3H). Twenty minutes after DI, overall gill morphology corresponded to that of untreated controls (not shown).

To identify the mechanisms of the observed cell death, gill filaments were examined ex vivo. Staining of gill filaments of untreated fish with PI, revealed a few PI^+ (necrotic) nuclei in the mucus layer surrounding the filaments (Fig. 4A). Three hours after HI, accumulations of PI^+ nuclei in the mucus layer were abundant (Fig. 4B). These accumulations were observed at least up to 24 h after HI (not shown). Filaments of none of the fish examined showed marked staining for the apoptosis marker annexin V (not shown).

3.3. Stress response

To examine the magnitude of the stress response evoked by HI and DI we measured plasma cortisol concentrations at various times after HI and compared these values with basal plasma cortisol concentrations (Fig. 5). Plasma cortisol concentrations peaked at 140 ng ml^{-1} around 20 min following a 2 min HI and returned to basal levels within 3 h after the immersion. Plasma cortisol levels following a 2 min sham immersion (normal tank water instead of 4.5% NaCl) peaked at 135 ng ml^{-1} at 20 min.

3.4. Phagocyte redistribution

To examine stress-induced neutrophilia after immunization with LPS–DTAF by HI and DI, we employed the mouse monoclonal antibody TCL-BE8. In circulation the percentage of TCL-$BE8^+$ cells displayed a sharp increase, from 4% in controls to almost 25% at 3 h after immersion vaccination (Fig. 6A, D and E). During the course of the first 48 h, percentages of TCL-$BE8^+$ cells gradually declined towards typical low values. The increase of circulating TCL-$BE8^+$ cells was accompanied by a concomitant decrease of these cells in the anterior kidney from 38% in controls to around 31% at 3 h after immersion (Fig. 6B). During the course of the experiment the percentage of TCL-$BE8^+$ cells in the anterior kidney slowly recovered to basal values. In gills the

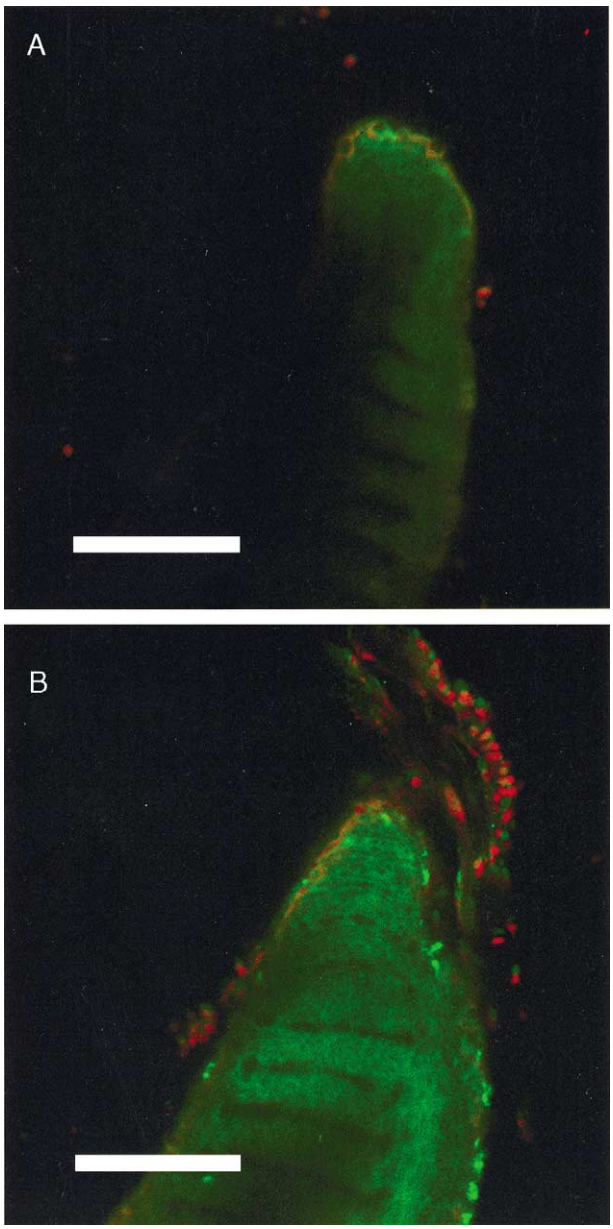

Fig. 4. Confocal laser scanning micrographs of carp gills stained as whole mount in vitro with PI. Occasional PI^+ nuclei could be observed in the mucus surrounding the tip of the filament of untreated fish (A). Three hours after HI (B), PI^+ nuclei had accumulated in the mucus. Scale bars: 100 μm, optical slices: 5.5 μm.

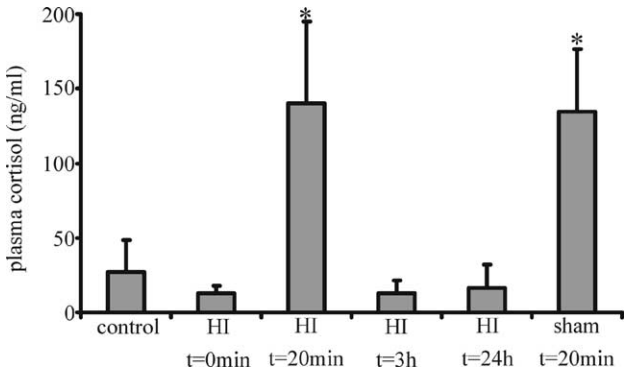

Fig. 5. Kinetics of plasma cortisol concentrations at various times after the end of HI and sham immersion. Error bars denote S.D. of 10 replicate measurements. Asterisk indicates a significant difference from the control ($P < 0.001$).

percentage of TCL-BE8$^+$ cells showed a marked increase 3 h after treatment (Fig. 6C), which appeared not merely to result from the increase of TCL-BE8$^+$ cells in circulation, as phagocytes were readily observed within the epithelium of the lamellae (Fig. 6F). Despite the higher increase of the percentage of TCL-BE8$^+$ cells in the gills 3 h after HI there was no statistically significant difference between both immersion protocols with respect to kinetics of phagocyte redistribution in any of the organs at any of the times tested. After immunization with BSA-FITC, kinetics of redistribution were comparable to those described for LPS–DTAF (not shown).

3.5. Antigen uptake

The uptake of a particulate antigen, FITC-labeled *A. salmonicida*, was invariably low, regardless of the immersion

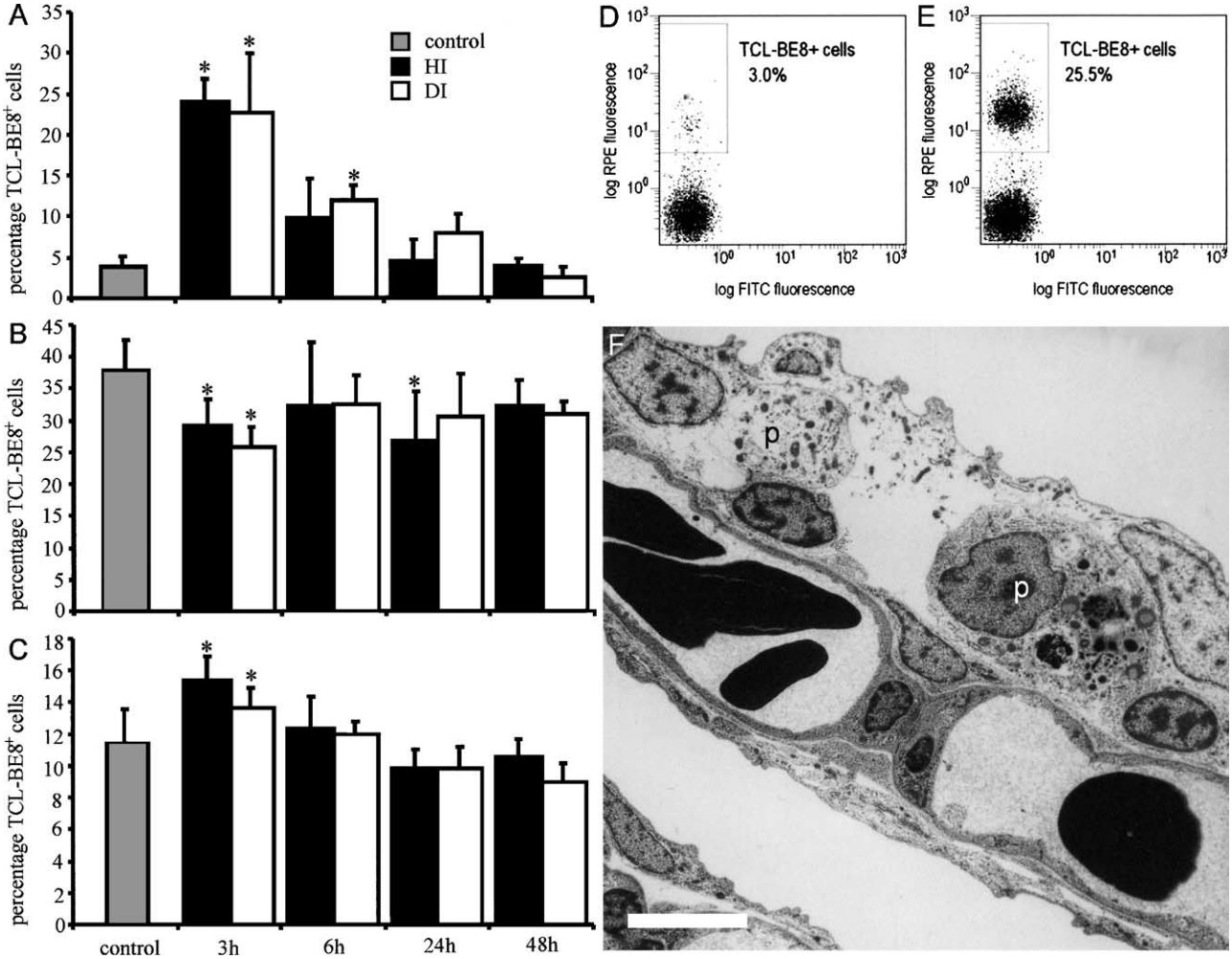

Fig. 6. The kinetics of percentages of TCL-BE8$^+$ cells in circulation (A), the anterior kidney (B) and the gills (C) after the indicated treatment. Error bars denote S.D. of six replicate measurements. Asterisk denotes a significant difference from the control ($P < 0.05$). Two representative dotplots of the percentages of TCL-BE8$^+$ cells in the circulation of a control fish (D) and of a fish 3 h following HI (E) are shown. Panel F shows an electron micrograph of two phagocytes (p), residing in the gill tissue after HI treatment. Scale bar: 5 μm.

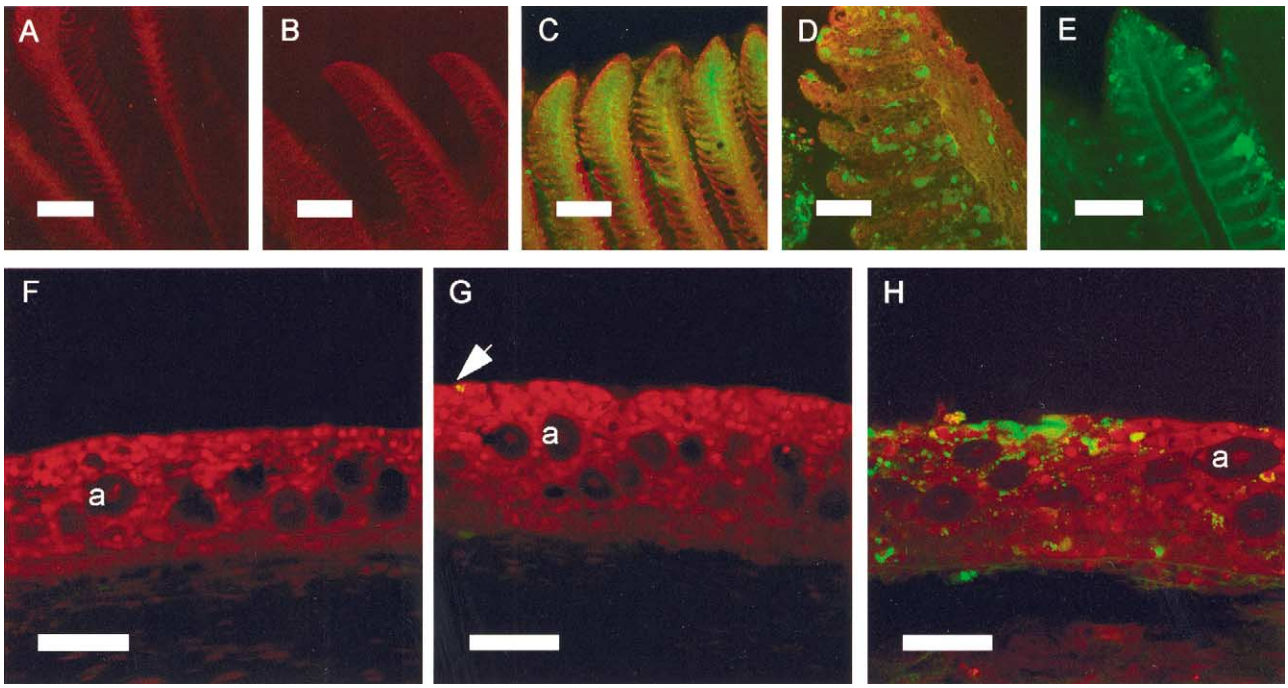

Fig. 7. Confocal laser scanning micrographs of uptake in carp gills of LPS–DTAF (green; A–D) and BSA-FITC (green; E) and LPS–DTAF in carp skin (F–H). Ten minutes after DI (B and G) uptake of LPS–DTAF (arrowhead) was observed, compared to control (A and F). Uptake of LPS–DTAF (C, D and H) and BSA-FITC (E) following HI was greatly enhanced compared to DI. PI (red) was applied as a general nuclear stain, except in panel E. a, alarm cell. Scale bars: (A–C) 200 μm; (E) 100 μm; (D, and F–G) 50 μm. Optical slices: (A–C) 14.9 μm; (E) 3.4 μm; (D, and F–G) 2.0 μm.

treatment. Bacteria adhered in considerable numbers to the surfaces of gills and, to a lesser extent to skin, but clear uptake within the tissue of these organs was rare (not shown).

LPS–DTAF was only marginally taken up in the gills and skin using DI (Fig. 7B and F). Application of HI greatly enhanced the amount of LPS–DTAF taken up as well as the penetration of antigen deeper into the tissue of skin and gills (Fig. 7C, D and G). A substantial portion of LPS–DTAF was taken up intracellularly in both epithelial cells (Fig. 8A) and in phagocyte-like cells residing deeper within the tissue of gills and skin (Fig. 8B and C), sometimes with prominent neutrophilic granulocyte-like features such as a lobular nucleus (Fig. 8B). Differences in the uptake of LPS–DTAF and BSA-FITC were not observed. Although some cells had taken up LPS–DTAF or BSA-FITC as early as 10 min after HI, at 6 h after HI BSA-FITC uptake was maximized. At that time 3.5% of the leucocytes collected by density gradient centrifugation was positive for BSA-FITC (Fig. 8D). About one-third of these cells was TCL-BE8$^+$ and therefore considered phagocytic. Significantly more cells had taken up BSA at 3 h after vaccination with HI compared to DI.

3.6. Activation of innate immunity

To assess activation of the innate immune system, an RQ-PCR assay was developed for relevant genes associated with acute inflammation. Interleukin-1β1 (IL-1β1; Fig. 9A; [43], but not tumor necrosis factor-α (Fig. 9E), was drastically upregulated in the hours following immersion vaccination with LPS–DTAF. This upregulation was observed after application of both vaccination protocols, but was more pronounced after HI. Ten minutes after the end of HI, expression of IL-1β1 had already increased 2.5-fold. Three hours after the end of HI the induction of IL-1β1 expression reached its peak at over 70-fold increase over control, to return to baseline values between 24 and 48 h after HI. The increase of expression of inducible NO synthase (iNOS) after HI followed similar kinetics and the peak induction at 3 h after HI was almost 4-fold (Fig. 9B). Application of DI only induced a modest increase in iNOS expression, 30 min after treatment. Expression of α_2-macroglobulin had increased 3 h after HI, reaching peak levels of 2.5-fold induction at 6 h after the end of HI (Fig. 9C). DI failed to increase expression of α_2M. Serum amyloid protein A (SAA) expression gradually increased to peak at an 11-fold increase at 24 h after HI (Fig. 9D). In the first 3 h there was no appreciable difference between the two treatments, as both caused an induction of SAA expression.

3.7. Specific mucosal immunity

As a measure for the amount of antigen-specific antibodies secreted into the mucus or present in the serum we used binding to LPS–DTAF, coated onto the surface of an optical

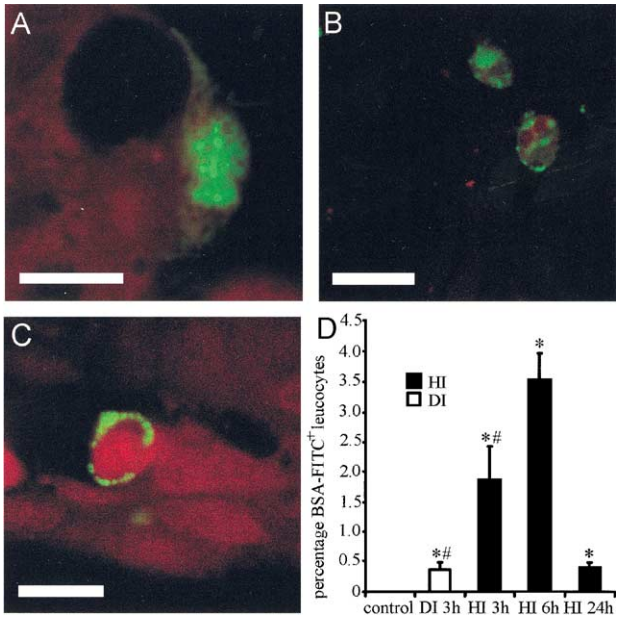

Fig. 8. Confocal laser scanning micrographs of intracellular uptake of LPS–DTAF (green). Panel A shows a gill epithelial cell, filled with LPS–DTAF. Panel B shows two LPS–DTAF$^+$ cells in the gills. Note the typical lobular nuclear shape of the lower cell. Panel C shows an LPS–DTAF$^+$ cell within the skin epidermis. Scale bars indicate 10 μm. Optical slices 1.2 μm. Panel D shows a relative quantification by flow cytometer of the kinetics of BSA-FITC$^+$ cells after the indicated treatment. Error bars denote S.D. of six replicate measurements. The control group never displayed FITC-positivity. Asterisk indicates a significant difference from the control ($P < 0.01$). # indicates a pairwise significant difference between two groups ($P < 0.01$).

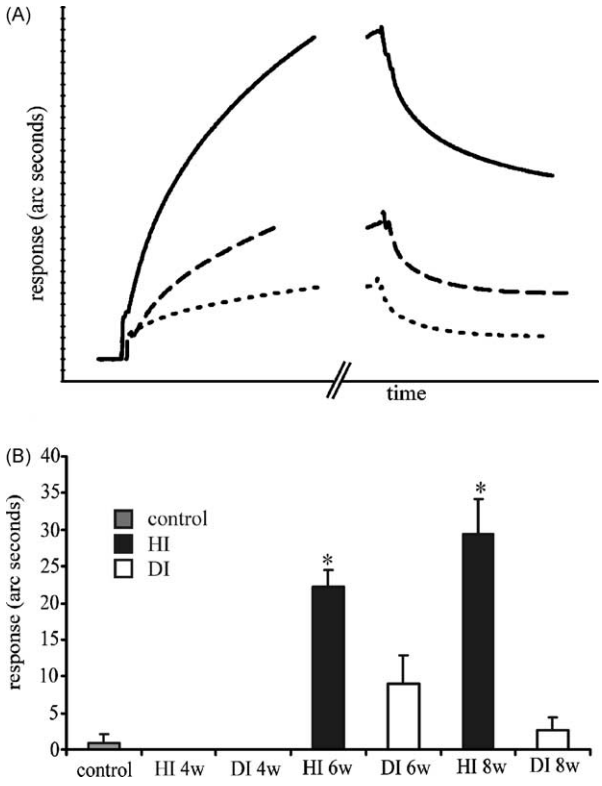

Fig. 10. Presence of LPS–DTAF specific binding detected by optical biosensor. Panel A shows three representative binding curves of mucus samples to an LPS–DTAF coated cuvette. The dotted line is from an unvaccinated fish. The dashed and solid lines are the responses detected in mucus of fish 8 weeks after DI and HI, respectively. Note that each curve consists of an association and a dissociation phase. The difference with the base line at the end of the dissociation phase (as determined by a fixed time) is taken as the readout parameter. These differences are plotted in panel B for each treatment. At 4 weeks there is no detectable response in any of the fish tested. Error bars indicate S.E. of the mean. Asterisks indicate a significant difference from the control ($P < 0.01$).

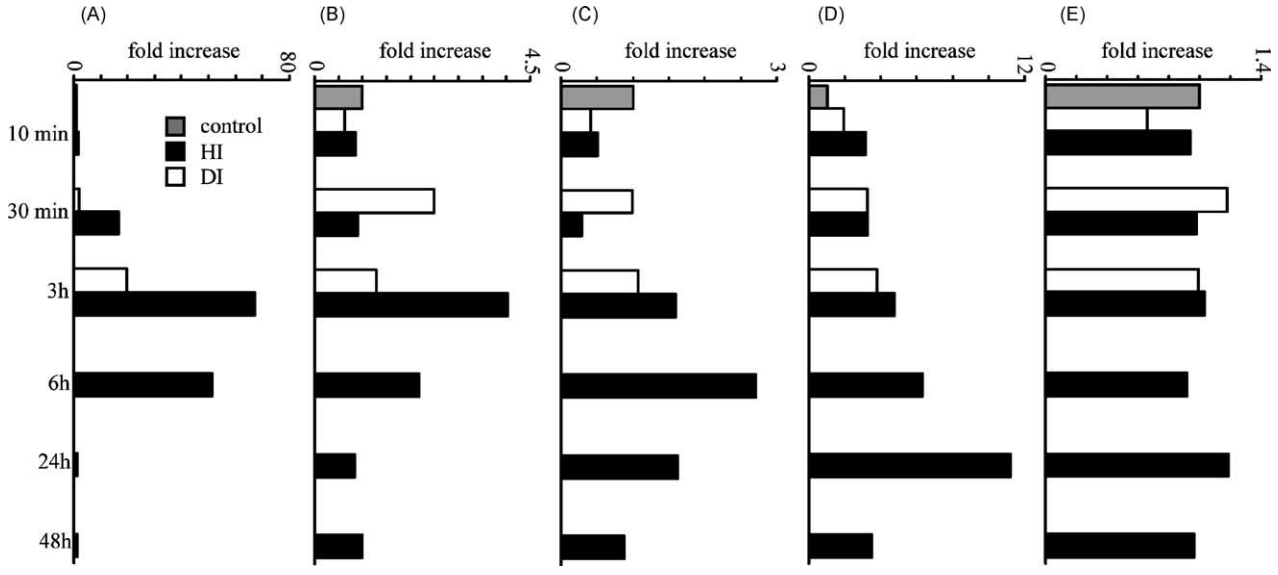

Fig. 9. RQ-PCR was performed to assess the expression of several acute inflammatory genes in gills after the indicated treatment. Expression was standardized for expression of 40S ribosomal protein S11 and relative to expression of controls. (A) IL-1β1, (B) iNOS, (C) α_2M, (D) SAA, (E) TNFα. Representative of two experiments is shown.

resonance cuvette. The same dilutions of mucus and serum from all fish were tested. The curves generated by this approach consisted of two phases (Fig. 10A): an association phase, in which binding to the coated surface was established, and a dissociation phase in which low affinity binding was removed, leaving only the higher affinity binding. The response of each sample was quantified as the difference between the curve at the end of the dissociation phase and the baseline, corrected for the bulk effect, which is the sudden increase of the base line at the moment a protein-rich sample is added. A detectable antigen-specific immune response was seen as of 6 weeks after HI (Fig. 10B). Eight weeks after HI, the response had increased further. The antigen-specific response following DI lagged behind the responses detected after HI, especially at 8 weeks after vaccination. At 8 weeks following HI, the response also displayed a higher affinity, as judged by the steeper association phase (Fig. 10A). No detectable antigen-specific serum immune response was detected at any of the times following either treatment.

4. Discussion

Immersion vaccination is standard procedure in fish aquaculture, but mechanisms of achieving maximal protection are far from elucidated. After comparison of two methods of immersion vaccination, it is concluded that HI appreciably enhances the overall immune response without placing a greater load on animal well being. We adapted the initial protocol of HI, developed for rainbow trout [19] to carp by lowering the osmolality of the hypertonic solution from 1650 mOsm kg^{-1} (5.3% (w/v) NaCl) to 1450 mOsm kg^{-1} (4.5% (w/v) NaCl). Initial pilot experiments indicated that there were no major differences in antigen uptake following these two protocols. This is corroborated by Fender and Amend, who showed that at an osmotic value between 1200 and 1400 mOsm kg^{-1} antigen uptake rises sharply and that a further increase of osmolality does not significantly increase antigen uptake [7].

The strong increase in plasma Na^+ and Cl^- concentrations and osmolality immediately following HI are a direct result of the hyperosmotic treatment. Apparently the stenohaline carp cannot cope with the sudden changes in salinity of this magnitude. Following HI however, when the fish had been returned to fresh water, the homeostatic equilibrium was quickly restored. The slight increase in plasma Na^+ and Cl^- concentrations observed at 3 h after HI are attributed to the stress response seen, as cortisol is known to elevate whole body influxes of Na^+ and Cl^- [44].

The extreme osmolality changes of the external milieu causes some epithelial cells to collapse under the osmotic pressure and undergo necrosis. These cells are quickly shed from the epithelium, leaving large holes that facilitate entry of soluble antigen greatly. However, uptake of particulate antigen, like inactivated *A. salmonicida* bacteria, is not enhanced appreciably, although the particle size is smaller than the size of the holes in the epithelium. Probably particulate antigen can only penetrate one cell layer into the tissue, as that is the extent of the damage inflicted by HI, whereas soluble antigen can penetrate deeper into the epithelial tissue once it has crossed the tight osmotic boundary of the outermost cell layer. The lack of appreciable uptake of particulate antigen suggests that it is the soluble moiety that is most important in inducing specific immunity following immersion vaccination. Recovery of the epithelial integrity was remarkably fast; within 20 min the epithelial holes were restored, despite some minor morphological differences from the normal situation, which take somewhat longer to restore. The accumulation of necrotic, i.e. PI^+ nuclei observed in the gills until at least 24 h after HI are mostly remnants of cells shed in the initial minutes after HI, that are retained within the mucus layer before being carried off altogether. Gill epithelium is a rapidly renewing tissue [45], a characteristic that ensures quick recovery from minor epithelial damage, which is an essential feature of a continuous barrier to the external milieu. This is beneficial to HI, as it minimizes the risk of opportunistic infections. However, in the initial minutes after HI the epithelium is leaky to any soluble component in the vaccine, including residual formalin present in formalin-inactivated bacterins. This is noteworthy since formalin is known to have pathological effects at the very low doses associated with many formalin-inactivated vaccines [46] and formalin might have a greater impact in combination with HI.

Recently immersion vaccination in combination with ultrasound has been proposed as a viable approach to enhance vaccination success [47]. We anticipate that short ultrasound exposure evokes ultrastructural alterations [48] not unlike those reported here, that have a similar effect in enhancing vaccine uptake as does HI. However, it is a considerably less accessible method in the field in comparison with HI.

The stress response induced by HI is a typical acute stress response, with peak plasma cortisol values around 20 min after treatment and returning to basal levels within hours [49]. Remarkably the peak cortisol levels measured at 20 min after both HI and sham immersion are very similar, indicating that it is the handling and restraint associated with immersion that form the actual stressor and that the osmotic challenge itself does not further contribute to the magnitude of the stress response. One could argue that peak cortisol levels are already maximized, but this is not the case, as considerably higher peak plasma cortisol values resulting from an acute stressor have been reported for the carp strain used [50].

Stress-induced neutrophilia is a well-known phenomenon. It is considered a healthy strategy to rapidly increase the number of circulating phagocytes, thereby temporarily enhancing peripheral surveillance. This is an adaptation to cope with the increased chances of injury and infection that are commonly associated with stress. In fish the anterior kidney is one of the most prominent haematopoietic organs and it is likely that phagocytes directed towards the periphery originate from the anterior kidney at least for a substantial part.

The osmotic shock itself does not appreciably contribute to the redistribution of TCL-BE8$^+$ cells from the anterior kidney towards the circulation, as the kinetics of TCL-BE8$^+$ cells following either HI or DI treatment are very similar. Therefore, the stress response is considered to be the driving force behind the redistribution of phagocytes into the circulation. The percentage of phagocytes in circulation has largely normalized within 24 h after the immersion vaccination, while the neutrophil population in the anterior kidney has not fully recovered within 48 h. Neutrophilic granulocytes, that constitute the larger portion of the redistributed phagocytes, are generally short-lived and either extravasate into peripheral tissues or are removed from the circulation by apoptosis [51,52]. Phagocyte populations in the systemic organs on the other hand are restored by cell proliferation, a process that takes days rather than hours to restore the population to normal.

From the circulation neutrophils migrate to peripheral organs such as gills and skin under the influence of locally secreted factors. This is a subtle effect, as the relative number of phagocytes in the gills only modestly increases following treatment. It is however not merely the result of the enhanced number of phagocytes in circulation, as active phagocytes are frequently observed within the tissue of the gill lamellae instead of within the capillary bed. We postulate that the slight difference in the percentage of TCL-BE8$^+$ cells between HI and DI 3 h after treatment is biologically significant and reflects enhanced local activation of the innate immune system. It is this enhanced redistribution towards the peripheral tissues in combination with the greatly increased uptake of soluble antigen over the epithelial boundary that accounts for the profound increase in the number of leukocytes that have taken up antigen. Since processing and presentation of antigen by professional antigen presenting cells is an essential step towards a specific humoral immune response, the redistribution of phagocytes towards the sites of antigen uptake is highly significant.

The rapid activation of local immunity is also evident from the acute upregulation of a number of acute phase factors. The cytokine IL-1β1 is one of the earliest cytokines released during inflammation [53]. Indeed we observe enhanced expression already at 30 min after the end of HI. Expression of iNOS is also rapidly induced, expression levels peaking at 3 h after the end of HI. NO synthesized by iNOS is a mediator of non-specific antimicrobial activities [54]. Peak levels in expression of α_2M and SAA lag behind those of IL-1β1 and iNOS. α_2M is an acute phase protein in many species, functioning both as a non-specific plasma protease inhibitor as well as a cytokine carrier [55]. In humans, α_2M is also implicated in induction of iNOS through neutralizing the anti-inflammatory cytokine transforming growth factor-β (TGFβ) [56] but this function is not likely in our case, as peak levels of iNOS expression precede those of α_2M. Not much is known of SAA but it is postulated to be involved in the repair of tissue damage [57]. Induction of SAA after injection of live *A. salmonicida* has been reported previously [58]. Surprisingly, TNFα expression is not upregulated, despite the fact that it is generally considered a pro-inflammatory cytokine that is often co-stimulated with IL-1β1 and is induced by *Escherichia coli* LPS [41], requiring further investigation. The differences with regard to the increase of expression of the various genes result from the different functions of these genes. IL-1β1 is a messenger, that has to carry its message over a considerable distance and therefore needs to be potently upregulated. iNOS, α_2M and SAA are downstream effectors involved in inflammation or recovery of tissue damage. Upregulation of these proteins does not need to be as robust to achieve maximal biological effect. The more profound activation of innate immunity caused by HI is likely caused by a combination of endogenous factors released upon local damage together with the substantial amount of LPS, which is known to be a potent stimulator of expression of IL-1β1 and iNOS [43,59]. The induction of IL-1β1 following DI and the comparable induction of SAA in the initial phase after both treatments is probably caused either, by uptake of small amounts of LPS–DTAF following DI, or by a more general mechanism, e.g. direct regulation by cortisol. The activation of innate immunity reported here is at least partially caused by the LPS we employed as a model vaccine and therefore may not be representative of most immersion vaccines. However, LPS and similar common pathogen motifs have been shown to constitute a large part of the soluble moiety of many vaccines. Bricknell et al. [60] showed that a humoral response to an extracellular polysaccharide of *A. salmonicida* was protective against challenge with virulent *A. salmonicida*. In a study of *Pasteurella piscicida* infection in gilthead seabream (*Sparus aurata*) a whole-cell bacterin did confer some protection only when enriched for the extracellular toxoid [61]. This suggests that at least for some pathogens the soluble moiety is crucial in inducing protective immunity.

The specific immune response resulting from immersion vaccination with 0.2% LPS–DTAF is detectable in skin mucus only, but not in plasma. This confirms the presence of a specific local immune system in fish, as suggested previously [12–15]. The exclusive induction of mucosal immunity following immersion vaccination fits in with the aim of protection to pathogens that commonly enter through the (external) mucosal surfaces. This confinement to the mucosal compartment may be the result of the relatively low doses of antigen used. Application of higher antigen doses through the same route may induce a ‘spill-over’ systemic response. The mucosal antibody response induced following HI is markedly stronger and lasts longer than that detected after DI. Moreover, the combined affinity of the response is higher following HI, suggesting affinity maturation. Taken together these data indicate the existence of a functional compartmentalization of the mucosal immune system, alike the mammalian situation, that can be selectively activated and achieve higher overall affinity.

Peak antibody levels following HI might lie beyond our final sampling time, which is 8 weeks after immersion

vaccination. However, the specific immune response takes quite long (at least 4 weeks) to develop to detectable levels. This is in line with previous reports on the detection of mucosal immunity by biosensor in rainbow trout (*Oncorhynchus mykiss*) [62]. Using an ELISPOT assay, numbers of antibody secreting cells in the gills of sea bass (*Dicentrarchus labrax*) peak at 8 days following DI with a bacterin of *Photobacterium damselae* ssp. *piscicida* [12]. The apparent lag between the peak responses may be attributed to differences in technique, as there may be a lag between the time of antibody secretion by specific plasma cells and the time that these antibodies are detectable in fish mucus.

In conclusion, we show that HI is a valid approach to boost specific immune responses through immersion vaccination. The main advantages of HI over DI are a dramatic increase in uptake of soluble antigen accompanied by a marked increase in the percentage of intracellular uptake by leucocytes. This initially leads to a profound acute inflammatory response and ultimately to an enhanced specific mucosal response. The damage inflicted by HI is instrumental to enhance uptake of soluble antigen and is rapidly repaired. The animals do not experience additional stress over the stress associated with handling. Essentially, HI acts as an adjuvant in the way it enhances vaccine uptake and stimulates the activation of the innate as well as the acquired immune system. Here, for the first time we thoroughly characterized the chain of events that is initiated by HI and that precedes ultimate specific mucosal immunity. Our data substantiate that HI makes a valuable contribution to enhancing the efficiency of immersion vaccination, making it an attractive alternative to injection methods. Furthermore, the data presented will enhance our understanding of fish vaccination and enable us to modify and improve vaccination protocols, so that maximal protection can be achieved with minimal costs and loss of production.

Acknowledgements

We thank Anja Taverne-Thiele and Coen van der Meij for excellent technical assistance. Huub Geurts for assistance with scanning electron microscopy and Jan van Lent for assistance with confocal microscopy. We thank Sietze Leenstra and Truus van der Wal of ‘De Haar Vissen’ and Tom Spanings of the fish facilities at the University of Nijmegen for taking care of the experimental animals. We acknowledge Dr. I.R. Bricknell for providing the *A. salmonicida* bacterin and LPS. Prof. N. Okamoto is acknowledged for providing the mouse monoclonal antibody TCL-BE8.

References

[1] Naylor RL, Goldburg RJ, Primavera JH, et al. Effect of aquaculture on world fish supplies. Nature 2000;405(6790):1017–24.
[2] Ellis AE. Immunization with bacterial antigens: furunculosis. In: Gudding R, Lillehaug A, Midtlyng PJ, Brown F, editors. Developments in Biological Standardization. Basel: Karger; 1997. p. 107–16.
[3] Horne MT. Technical aspects of the administration of vaccines. In: Gudding R, Lillehaug A, Midtlyng PJ, Brown F, editors. Developments in Biological Standardization. Basel: Karger; 1997. p. 79–89.
[4] Nakanishi T, Ototake M. Antigen uptake and immune responses after immersion vaccination. In: Gudding R, Lillehaug A, Midtlyng PJ, Brown F, editors. Developments in Biological Standardization. Basel: Karger; 1997. p. 59–68.
[5] Joosten PHM, Tiemersma E, Threels A, Caumartin Dhieux C, Rombout JHWM. Oral vaccination of fish against *Vibrio anguillarum* using alginate microparticles. Fish Shellfish Immunol 1997;7(7):471–85.
[6] Campbell R, Adams A, Tatner MF, Chair M, Sorgeloos P. Uptake of *Vibrio anguillarum* vaccine by *Artemia salina* as a potential oral delivery system to fish fry. Fish Shellfish Immunol 1993;3:451–9.
[7] Fender DC, Amend DF. Hyperosmotic infiltration: factors influencing uptake of bovine serum albumin by rainbow trout (*Salmo gairdneri*). J Fish Res Board Can 1978;35:871–4.
[8] Tatner MF. The quantitative relationship between vaccine dilution, length of immersion time and antigen uptake, using a radioloabelled *Aeromonas salmonicida* bath in direct immersion experiments with rainbow trout, *Salmo gairdneri*. Aquaculture 1987;62:173–85.
[9] Lillehaug A, Ramstad A, Baekken K, Reitan LJ. Protective immunity in Atlantic salmon (*Salmo salar* L.) vaccinated at different water temperatures. Fish Shellfish Immunol 1993;3:143–56.
[10] Tatner MF, Manning MJ. The ontogeny of cellular immunity in the rainbow trout, *Salmo gairdneri* Richardson, in relation to the stage of development of the lymphoid organs. Dev Comp Immunol 1983;7(1):69–75.
[11] Zapata AG, Torroba M, Varas A, Jimenez AV. Immunity in fish larvae. In: Gudding R, Lillehaug A, Midtlyng PJ, Brown F, editors. Developments in Biological Standardization. Basel: Karger; 1997. p. 23–32.
[12] dos Santos NM, Taverne-Thiele JJ, Barnes AC, van Muiswinkel WB, Ellis AE, Rombout JH. The gill is a major organ for antibody secreting cell production following direct immersion of sea bass (*Dicentrarchus labrax*, L.) in a *Photobacterium damselae* ssp. *piscicida* bacterin: an ontogenetic study. Fish Shellfish Immunol 2001;11(1):65–74.
[13] Lumsden JS, Ostland VE, Byrne PJ, Ferguson HW. Detection of a distinct gill-surface antibody response following horizontal infection and bath challenge of brook trout *Salvelinus fontinalis* with *Flavobacterium branchiophilum*, the causative agent of bacterial gill disease. Dis Aquat Organ 1993;16(1):21–7.
[14] Gudding R, Lillehaug A, Evensen O. Recent developments in fish vaccinology. Vet Immunol Immunopathol 1999;72(1–2):203–12.
[15] Lobb CJ. Secretory immunity induced in catfish, *Ictalurus punctatus*, following bath immunization. Dev Comp Immunol 1987;11(4):727–38.
[16] Ototake M, Iwama George K, Nakanishi T. The uptake of bovine serum albumin by the skin of bath-immunised rainbow trout *Oncorhynchus mykiss*. Fish Shellfish Immunol 1996;6(5):321–33.
[17] Kiryu I, Ototake M, Nakanishi T, Wakabayashi H. The uptake of fluorescent microspheres into the skin, fins and gills of rainbow trout during immersion. Fish Pathol 2000;35(1):41–8.
[18] Tatner MF, Johnson CM, Horne MT. The tissue localization of *Aeromonas salmonicida* in rainbow trout, *Salmo gairdneri* Richardson, following three methods of administration. J Fish Biol 1984;25:95–108.
[19] Amend DF, Fender DC. Uptake of bovine serum albumin by rainbow trout from hypersmotic solutions: a model for vaccinating fish. Science 1976;192(4241):793–4.
[20] Robohm RA, Koch RA. Evidence for oral ingestion as the principal route of antigen entry in bath-immunized fish. Fish Shellfish Immunol 1995;5(2):137–50.

[21] Tatner MF, Horne MT. Factors influencing the uptake of 14C-labelled *Vibrio anguillarum* vaccine in direct immersion experiments with rainbow trout, *Salmo gairdneri* Richardson. J Fish Biol 1983;22:585–91.

[22] Moore JD, Ototake M, Nakanishi T. Particulate antigen uptake during immersion immunisation of fish: the effectiveness of prolonged exposure and the roles of skin and gill. Fish Shellfish Immunol 1998;8(6):393–407.

[23] Anderson DP, Roberson BS, Dixon OW. Plaque-forming cells and humoral antibody in rainbow trout (*Salmo gairdneri*) induced by immersion in a *Yersina ruckeri* O-antigen preparation. J Fish Res Board Can 1979;36:636–9.

[24] Tatner MF, Horne MT. Correlation of immune assays with protection in rainbow trout, *Salmo gairdneri*, immersed in *Vibrio* bacterins. J Appl Ichtyol 1986;3:130–9.

[25] Mughal MS, Farley-Ewens EK, Manning MJ. Effects of direct immersion in antigen on immunological memory in young carp, *Cyprinus carpio*. Vet Immunol Immunopathol 1986;12(1–4):181–92.

[26] Zapata AG, Torroba M, Alvarez F, Anderson DP, Dixon OW, Wisniewski M. Electron microscopic examination of antigen uptake by salmonid gill cells after bath immunization with a bacterin. J Fish Biol 1987;31(Suppl A):209–17.

[27] Antipa R, Gould R, Amend DF. *Vibrio anguillarum* vaccination of sockeye salmon *Oncorhynchus nerka* (Walbaum) by direct and hyperosmotic immersion. J Fish Dis 1980;3(2):161–5.

[28] Liewes EW, van Dam RH, Vos-Maas MG, Bootsma R. Presence of antigen sensitized leukocytes in carp (*Cyprinus carpio* L.) following bath immunization against *Flexibacter columnaris*. Vet Immunol Immunopathol 1982;3(6):603–9.

[29] Lumsden JS, Ostland VE, MacPhee DD, Ferguson HW. Production of gill-associated and serum antibody by rainbow trout (*Oncorhynchus mykiss*) following immersion immunization with acetone-killed *Flavobacterium branchiophilum* and the relationship to protection from experimental challenge. Fish Shellfish Immunol 1995;5:151–65.

[30] Thuvander A, Hongslo T, Jansson E, Sundquist B. Duration of protective immunity and antibody titres measured by ELISA after vaccination of rainbow trout, *Salmo gairdneri* Richardson, against vibriosis. J Fish Dis 1987;10:479–86.

[31] Whittington RJ, Munday BL, Akhlaghi M, Reddacliff GL, Carson J. Humoral and peritoneal cell responses of rainbow trout (*Oncorhynchus mykiss*) to ovalbumin, *Vibrio anguillarum* and Freund's complete adjuvant following intraperitoneal and bath immunisation. Fish Shellfish Immunol 1994;4:475–88.

[32] Baba T, Imamura J, Izawa K, Ikeda K. Immune protection in carp, *Cyprinus carpio* L., after immunization with *Aeromonas hydrophila* crude lipopolysaccharide. J Fish Dis 1988;11(3):237–44.

[33] Hirst ID, Ellis AE. Iron-regulated outer membrane proteins of *Aeromonas salmonicida* are important protective antigens in Atlantic salmon against furunculosis. Fish Shellfish Immunol 1994;4:29–45.

[34] Irnazarow I. Genetic variability of Polish and Hungarian carp lines. Aquaculture 1995;129:215–9.

[35] Ellis AE, Burrows AS, Stapleton KJ. Lack of relationship between virulence of *Aeromonas salmonicida* and the putative virulence factors: a layer, extracellular proteases and extracellular haemolysins. J Fish Dis 1988;11:309–23.

[36] de Belder AE, Granath K. Preparation and properties of fluorescein-labelled dextrans. Carbohydr Res 1973;30(2):375–8.

[37] Hudson L, Hay FC. Practical Immunology. Oxford: Blackwell Scientific Publications; 1989. p. 507.

[38] Balm PH, Pepels P, Helfrich S, Hovens ML, Bonga SE. Adrenocorticotropic hormone in relation to interrenal function during stress in tilapia (*Oreochromis mossambicus*). Gen Comp Endocrinol 1994;96(3):347–60.

[39] Nakayasu C, Omori M, Hasegawa S, Kurata O, Okamoto N. Production of a monoclonal antibody for carp (*Cyprinus carpio* L.) phagocytic cells and separation of the cells. Fish Shellfish Immunol 1998;8(2):91–100.

[40] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 1987;162(1):156–9.

[41] Saeij JP, Stet RJ, de Vries BJ, van Muiswinkel WB, Wiegertjes GF. Molecular and functional characterization of carp TNF: a link between TNF polymorphism and trypanotolerance? Dev Comp Immunol 2003;27(1):29–41.

[42] Applied Biosystems. User Bulletin #2. ABI Prism 7700. Sequence Detection System; 2001. p. 36.

[43] Engelsma MY, Stet RJ, Schipper H, Verburg-van Kemenade BM. Regulation of interleukin 1 beta RNA expression in the common carp, *Cyprinus carpio* L.. Dev Comp Immunol 2001;25(3):195–203.

[44] Laurent P, Perry SF. Effects of cortisol on gill chloride cell morphology and ionic uptake in the freshwater trout, *Salmo gairdneri*. Cell Tissue Res 1990;259:429–42.

[45] Lyndon AR, Houlihan DF. Gill protein turnover: costs of adaptation. Comp Biochem Physiol A Mol Integr Physiol 1998;119(1):27–34.

[46] Cruz ER, Pitogo CL. Tolerance level and histopathological response of milkfish (*Chanos chanos*) fingerlings to formalin. Aquaculture 1989;78:135–45.

[47] Fernandez-Alonso M, Rocha A, Coll JM. DNA vaccination by immersion and ultrasound to trout viral haemorrhagic septicaemia virus. Vaccine 2001;19(23–24):3067–75.

[48] Frenkel V, Kimmel E, Iger Y. Ultrasound-induced intercellular space widening in fish epidermis. Ultrasound Med Biol 2000;26(3):473–80.

[49] Weyts FA, Verburg-van Kemenade BM, Flik G, Lambert JG, Wendelaar Bonga SE. Conservation of apoptosis as an immune regulatory mechanism: effects of cortisol and cortisone on carp lymphocytes. Brain Behav Immun 1997;11(2):95–105.

[50] Tanck MWT, Booms GHR, Eding EH, Wendelaar Bonga SE, Komen J. Cold shocks: a stressor for common carp. J Fish Biol 2000;57(4):881–94.

[51] Ruiz LM, Bedoya G, Salazar J, de Garcia OD, Patino PJ. Dexamethasone inhibits apoptosis of human neutrophils induced by reactive oxygen species. Inflammation 2002;26(5):215–22.

[52] Watson RW. Redox regulation of neutrophil apoptosis. Antioxid Redox Signal 2002;4(1):97–104.

[53] Engelsma MY, Huising MO, van Muiswinkel WB, et al. Neuroendocrine-immune interactions in fish: a role for interleukin-1. Vet Immunol Immunopathol 2002;87(3–4):467–79.

[54] Nathan C, Shiloh MU. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc Natl Acad Sci USA 2000;97(16):8841–8.

[55] Webb DJ, Wen J, Lysiak JJ, Umans L, van Leuven F, Gonias SL. Murine alpha-macroglobulins demonstrate divergent activities as neutralizers of transforming growth factor-beta and as inducers of nitric oxide synthesis: a possible mechanism for the endotoxin insensitivity of the alpha2-macroglobulin gene knock-out mouse. J Biol Chem 1996;271(40):24982–8.

[56] Lysiak JJ, Hussaini IM, Webb DJ, Glass II WF, Allietta M, Gonias SL. Alpha 2-macroglobulin functions as a cytokine carrier to induce nitric oxide synthesis and cause nitric oxide-dependent cytotoxicity in the RAW 264.7 macrophage cell line. J Biol Chem 1995;270(37):21919–27.

[57] Bayne CJ, Gerwick L. The acute phase response and innate immunity of fish. Dev Comp Immunol 2001;25(8–9):725–43.

[58] Jensen LE, Hiney MP, Shields DC, Uhlar CM, Lindsay AJ, Whitehead AS. Acute phase proteins in salmonids: evolutionary analyses and acute phase response. J Immunol 1997;158(1):384–92.

[59] Saeij JP, Stet RJ, Groeneveld A, Verburg-van Kemenade BM, van Muiswinkel WB, Wiegertjes GF. Molecular and functional characterization of a fish inducible-type nitric oxide synthase. Immunogenetics 2000;51(4–5):339–46.
[60] Bricknell IR, Bowden TJ, Lomax J, Ellis AE. Antibody response and protection of Atlantic salmon (*Salmo salar*) immunised with an extracellular polysaccharide of *Aeromonas salmonicida*. Fish Shellfish Immunol 1997;7(1):1–16.
[61] Magarinos B, Romalde JL, Santos Y, Casal JF, Barja JL, Toranzo AE. Vaccination trials on gilthead seabream (*Sparus aurata*) against *Pasteurella piscicida*. Aquaculture 1994;120:201–8.
[62] Cain KD, Jones DR, Raison RL. Characterisation of mucosal and systemic immune responses in rainbow trout (*Oncorhynchus mykiss*) using surface plasmon resonance. Fish Shellfish Immunol 2000;10(8):651–66.