

# Cytokine signalling in rat pulp interstitial fluid and transcapillary fluid exchange during lipopolysaccharide-induced acute inflammation

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The dental pulp consists of loose connective tissue encased in rigid dentinal walls. Because of its topography the tissue has low interstitial compliance and limited capacity to expand during fluid volume changes. Due to limitations regarding access to interstitial fluid, basic knowledge on transcapillary fluid transport parameters is lacking for this organ. The scope of this project was dual: first we aimed at establishing a method for isolation of pulp interstitial fluid (IF), and second we applied the method in rats subjected to lipopolysaccharide (LPS)-induced endotoxaemia. The aim was to measure colloid osmotic pressure (COP) and pro-inflammatory cytokines in the pulp IF during acute inflammation. Fluid volumes and pulpal blood flow (PBF) were measured to obtain more information about microcirculatory changes that take place in this pulpitis model. By centrifugation of incisor pulp at 239 g we were able to extract fluid representative for IF. Pulp IF had a relative high control COP ( $\sim 83\%$  of plasma COP) and was similar to plasma COP 3 h after LPS challenge. The pulp exhibited a high content of IF ( $0.60 \pm 0.03$  ml (g wet weight)<sup>-1</sup>) and a vascular volume of  $0.03 \pm 0.01$  ml (g w.w.)<sup>-1</sup>. No differences were observed in the distribution of fluid volumes after 1.5 and 3 h LPS exposure. PBF and systemic blood pressure dropped significantly after LPS administration. PBF remained low whereas systemic blood pressure was re-established during the 3-h period, implying organ dysfunction. There was a differential pattern of cytokine expression in pulp IF and serum with cytokines such as IL-1 $\alpha$ , IL-1 $\beta$  and TNF- $\alpha$  locally produced, whereas others such as IFN- $\gamma$  and IL-6 were produced systemically and probably spilled over to the pulp IF after LPS exposure. Our findings show that pulp IF can be isolated by centrifugation and that this method is useful when studying fluid balance and extracellular signalling mechanisms in the dental pulp in normal and pathological conditions.

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The dental pulp is a highly vascular connective tissue enclosed in the rigid mineralized dentin. It shares many similarities with other connective tissues of the body but it also has circulatory characteristics with physiological implications. The pulp is a microcirculatory system lacking collateral circulation, and is situated within a low-compliance environment similar to, e.g. the brain. The limited ability to expand may severely compromise the circulation under conditions with increased fluid volume. The above features render the pulp vulnerable to circulatory changes occurring in inflammation such as hyperaemia and increased fluid filtration.

Pulpitis may be painful and is a very common inflammatory condition in man, usually caused by carious bacteria (Sindet-Pedersen *et al.* 1985; Khabbaz *et al.* 2001; Morgan *et al.* 2005). The first vascular reactions during pulpitis are vasodilatation and increased vascular permeability (Kerezoudis *et al.* 1993; Heyeraas *et al.* 1994). The observed increase in interstitial fluid pressure (Heyeraas & Berggreen, 1999) suggests that there is an increased interstitial fluid volume in this situation that will counteract further fluid filtration, but there are, however, no data available concerning intra- and extra-vascular fluid volumes either in normal or inflamed pulp. Furthermore, increased vascular permeability may again induce changes in colloid osmotic pressure of the interstitial fluid (COP<sub>i</sub>), another key factor in

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transcapillary flow according to Starling's equation. Up to now,  $COP_i$  in normal as well as in inflamed pulp is unknown.

Since the dental pulp is enclosed in hard dentinal walls, direct access to the pulp tissue is difficult without exposing the pulp tissue and thereby creating inflammation. Using a recent technique applied in tumours and skin (Wiig *et al.* 2003) we tested if this method could be used for isolation of interstitial fluid (IF) from tooth pulp. We centrifuged pulp tissue at 239 g and isolated pulp fluid to determine local levels of pro-inflammatory cytokines during the development of acute pulpitis in order to assess the role of extracellular signalling in the microenvironment surrounding the pulpal cells. We chose a model of sepsis by administration of lipopolysaccharide (LPS) through the vascular system to achieve widespread pulpitis in the rats, as LPS has been implicated in the pathogenesis of pulpitis by entering the pulp via dentinal tubules (Warfvinge *et al.* 1985). The concentrations of six among the most investigated pro-inflammatory cytokines (interleukin-1 $\alpha$  (IL-1 $\alpha$ ), IL-1 $\beta$ , IL-2, IL-6, interferon- $\gamma$  (IFN- $\gamma$ ) and tumour necrosis factor  $\alpha$  (TNF- $\alpha$ )) were measured in both pulp IF and serum, to test the hypothesis that LPS can cause local production and release of cytokines from pulpal cells. Furthermore,  $COP_i$  and fluid volume measurements were performed after LPS challenge to obtain more information about transcapillary fluid balance during pulpitis. In addition, pulpal blood flow (PBF) was measured continuously during LPS exposure to observe the micro-circulatory changes that take place in this model of acute inflammation.

## Methods

### Experimental animals

The experiments were performed in intraperitoneally anaesthetized (50 mg kg<sup>-1</sup> sodium pentobarbital, Svaneapoteket, Bergen, Norway) female Wistar rats ( $n = 69$ , 190–220 g body weight). A femoral vein was catheterized (polyethylene PE-50 catheter) for injection of supplemental anaesthetic (2–3 mg kg<sup>-1</sup> i.v.) and substances, and a femoral artery for continuous systemic blood pressure (PA) recordings with a Gould pressure transducer and recorder (RS 3400; Cleveland, OH, USA). Body temperature was kept at 37–38°C with a servo-controlled heating pad. The depth of anaesthesia was assessed by the absence of spontaneous eye movements and foot or tail withdrawal in response to pinch and supplemental anaesthesia was given when necessary. At the end of the experiments the animals were killed with 0.5 ml saturated potassium chloride (KCl) i.v. All experiments were performed in accordance with recommendations given by the Norwegian State Commission for Laboratory Animals and were approved by the local ethical committee.

### Measurements of fluid volumes

Twenty rats were used for fluid volume measurements. Total extracellular fluid volume ( $V_x$ ) and intravascular volume ( $V_v$ ) were measured as the distribution volumes of <sup>51</sup>Cr-labelled EDTA and <sup>125</sup>I-labelled human serum albumin (HSA), respectively. Following anaesthesia and placement of catheters, both kidney pedicles were ligated via flank incisions, and 30  $\mu$ Ci of <sup>51</sup>Cr-EDTA was injected i.v. After a 120 min equilibration period, 3–4  $\mu$ Ci of <sup>125</sup>I-HSA was given i.v. and allowed to circulate for 5 min. A blood sample of 0.5–0.7 ml was obtained from the arterial catheter and the rat was killed. A small area at the back of the rat was shaved and a 2 cm  $\times$  2 cm piece of skin was cut with scissors and placed in a preweighed airtight tube. The rat was transferred to an incubator kept at room temperature (20–24°C) and 100% relative humidity. The four incisor teeth were removed and cracked and the pulp was taken out in one piece, and transferred to preweighed airtight tubes in order to avoid evaporation of fluid from the tissue. All tubes with samples were reweighed to obtain wet weight (w.w.) of the tissues. The blood samples were centrifuged at 11000 r.p.m. (12839 g) for 15 min. Known volumes of plasma were removed and used for further analysis. Samples (plasma, pulp and skin) were counted in an LKB  $\gamma$ -counter (Wallac 1282 Compugamma, Turku, Finland) using window settings of 15–75 keV for <sup>125</sup>I and 290–350 keV for <sup>51</sup>Cr. Standards were counted in every experiment to obtain spillover corrections and counts were corrected for background and spillover.

Fluid volumes were calculated as the plasma equivalent distribution volumes of the tracers, assuming that labelled EDTA (<sup>51</sup>Cr-EDTA) will distribute in the extracellular fluid phase and labelled HSA (<sup>125</sup>I-HSA) will distribute only in plasma. Intravascular plasma volume in a tissue sample ( $V_v$ ) was calculated as the 5 min distribution volume of <sup>125</sup>I-HSA:

$$V_v(\text{ml g}^{-1}) = \frac{\text{Counts } ^{125}\text{I-HSA/g tissue}}{\text{Counts } ^{125}\text{I-HSA/ml terminal plasma}} \quad (1)$$

Since <sup>125</sup>I-HSA was circulating in the animal for only 5 min, extravasation was assumed to be negligible. Tissue extracellular fluid volume ( $V_x$ ) was calculated as the 2 h distribution volume of <sup>51</sup>Cr-EDTA:

$$V_x(\text{ml g}^{-1}) = \frac{\text{Counts } ^{51}\text{Cr-EDTA/g tissue}}{\text{Counts } ^{51}\text{Cr-EDTA/ml terminal plasma}} \quad (2)$$

The tissue IF volume was calculated as the difference between extracellular fluid and plasma volume:

$$V_i(\text{ml g}^{-1}) = V_x - V_v \quad (3)$$

### Isolation of interstitial fluid

To isolate pulp IF we tested whether an approach described by Wiig *et al.* (2003) was applicable for this organ. The rats ( $n = 12$ ) were anaesthetized and nephrectomized as described above. Sixty to seventy microcuries of  $^{51}\text{Cr}$ -EDTA was injected i.v. After a 120 min equilibration period, 3–4  $\mu\text{Ci}$  of  $^{125}\text{I}$ -HSA was injected and allowed to circulate for 5 min. Blood samples and pulp tissues from the incisors were obtained the same way as previously described. The pulp was transferred to centrifuge tubes provided with a basket of nylon mesh with pore size  $\sim 15 \mu\text{m} \times 20 \mu\text{m}$  designed to keep the sample away from the bottom of the tube (Aukland, 1991), and spun in an Eppendorff 5417 R centrifuge for 10 min at 239 g. Thereafter the tubes were transferred back in the incubator and the fluid accumulated at the bottom of the tubes was collected in graded glass microcapillaries for volume measurements. Samples of 0.5–5  $\mu\text{l}$  of pulp IF could then be collected.

To examine if fluid was derived from the intracellular compartment, the ratio ( $R$ ) of the extracellular tracer  $^{51}\text{Cr}$ -labelled EDTA between isolated fluid and plasma was measured:

$$R = \frac{\text{Counts } ^{51}\text{Cr-EDTA/ml fluid}}{\text{Counts } ^{51}\text{Cr-EDTA/ml terminal plasma}} \quad (4)$$

$R < 1$  would indicate that fluid not containing the tracer is added to the centrifugate and opposed,  $R > 1$  would indicate that the extracellular fluid is concentrated, e.g. by cell swelling. The intravascular tracer  $^{125}\text{I}$ -HSA was used for determining contribution of fluid from the vascular compartment.

The distribution of macromolecules in isolated pulp fluid, pulp eluate, pulp tissue extract (homogenate) and plasma was determined by high performance liquid chromatography (HPLC). The pulp eluate was prepared by soaking intact pulp in phosphate buffer saline (PBS) for 6 h at 4°C. A whole pulp extract was obtained by cutting with micro-scissors, freeze-drying and crushing the pulp and soaking the minced tissue in PBS overnight at 4°C. After centrifugation the supernatant was collected for further analysis. For the HPLC we used two 4.6 mm (i.d.)  $\times$  30.0 cm TSK-gel size-exclusion columns coupled in series (Super SW2000 and 3000, Tosoh Biosciences, Stuttgart, Germany) with an optimal separation range for globular proteins of 5–100 kDa and 10–500 kDa, respectively. The protein concentration in the elution fluid was measured by UV detection at 220 nm on an Ettan<sup>TM</sup> LC System (GE Healthcare) and the buffer/mobile phase was 0.1 M  $\text{Na}_2\text{SO}_4$  in 0.1 M PBS, pH 7.0.

### Blood-flow recordings

A Periflux Model 4001 Master laser-Doppler flow meter (Perimed KB; Järfälla, Sweden) equipped with a

needle-probe PF 415:10 (fibre diameter 125  $\mu\text{m}$  with separation of 500  $\mu\text{m}$ ) was used to measure pulp blood flux proportional to local changes in blood flow in the left maxillary incisor tooth of representative animals that were treated 3 h with LPS. The head of the rat was immobilized and fixed to the operating table by a stereotaxic frame with the rat lying on its right side. The lips were pulled gently with thread in order to increase accessibility to the incisor tooth. The laser probe was positioned 3–5 mm above the level of the gingiva on the distal aspect of the tooth which gave the largest resting blood flow signal. Zero blood flow was determined as the value recorded from a stationary white card with the same intensity of reflected light as was measured when recording from the tooth. The flow meter's time constant was set at 0.03 s with an upper bandwidth of 20 kHz.

### Experimental protocol of LPS-induced acute inflammation

LPS from *Escherichia coli* 0127:B8 (Sigma-Aldrich Chemie, Schnellendorf, Germany) was dissolved in 0.9% NaCl containing 0.1% bovine serum albumin (BSA, Fraction V; Sigma) to a final concentration of 2.5 mg ml<sup>-1</sup>.

The rats received a dose of 4.0 mg kg<sup>-1</sup> LPS i.v. and were observed for 1.5 h (1.5 h LPS group,  $n = 6$ ) or 3 h (3 h LPS group,  $n = 6$ ). Control rats ( $n = 8$ ) received the equivalent volume of vehicle (0.9% NaCl with 0.1% BSA) and were kept for 1.5 h ( $n = 4$ ) or 3 h ( $n = 4$ ). In order to quantify cytokines in the pulp microenvironment, cytokine levels in pulp IF and serum from the above animals were determined with the multiplex assay. In addition, two more rats treated with LPS or vehicle for 3 h were used for immunohistochemical analysis of the pulp tissue.

For fluid volume measurements, the rats received 4 mg kg<sup>-1</sup> LPS after the 120 min equilibration period of  $^{51}\text{Cr}$ -EDTA. One and a half hours ( $n = 6$ ) or 3 h ( $n = 5$ ) after LPS administration, the intravascular marker  $^{125}\text{I}$ -HSA was given and the rats were killed 5 min later. The control group ( $n = 9$ ) received only the extracellular and the intravascular isotopes as previously described (measurement of fluid volumes).

Measurements of colloid osmotic pressure were performed in samples from rats treated with LPS for 3 h ( $n = 8$ ) and in seven controls. When possible, measurements of COP (from two incisors) were combined with measurements of fluid volumes (the other two incisors).

### Measurements of colloid osmotic pressure

After isolation of pulp IF and plasma, the COP ( $n = 15$ ) was measured in a colloid osmometer designed for sub-microlitre samples, using membranes with a cut-off size of 30 kDa (Wiig *et al.* 1988).

### Analysis of cytokines in IF and serum

Pulp IF and serum from 20 rats not given isotopes were isolated following the procedures described earlier. The graded microcapillaries with IF and isolated serum were stored at  $-80^{\circ}\text{C}$  until multiplex cytokine assay was performed.

IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-6, TNF- $\alpha$  and IFN- $\gamma$  were measured simultaneously in pulp fluid or serum using a Lincoplex kit (Linco Research, St Charles, MO, USA) according to the manufacturer's instructions. This kit offers a multiplexed microsphere-based flow cytometric immunoassay using the Luminex technology (Luminex Corporation Austin, TX, USA). A broad range of standards (4.8–20 000 pg ml $^{-1}$ ) was provided in the multiplex kit and the multiplexed assay was analysed on a flow cytometer (Luminex<sup>100</sup>, Luminex Corporation).

### Immunohistochemistry

Incisor teeth from rats exposed to LPS for 3 h and from control rats (3 h vehicle) were removed and split, and the pulp tissue was fixed in 4% paraformaldehyde with 0.2% picric acid. The pulp tissue was then rinsed in 0.1 M phosphate buffer, soaked in 30% sucrose overnight and stored at  $-80^{\circ}\text{C}$  until sectioning. The frozen specimens were then embedded in Tissue-Tek OCT compound (Sakura, Zoeterwoude, Netherlands), and 25  $\mu\text{m}$  thick sagittal sections were cut in a freezing ( $-20^{\circ}\text{C}$ ) slide microtome. The immunoreactions were performed on precoated glass slides (SuperFrost Plus, MenzelGlaser, Braunschweig, Germany). Alternate serial sections were incubated for 72 h in rat IL-1 $\alpha$  (dilution 1 : 400, Endogen, MA, USA) or TNF- $\alpha$  (dilution 1 : 300, Endogen) polyclonal antibodies raised in rabbit, at  $4^{\circ}\text{C}$ . The specificity of the immune reaction was tested by omission of the primary antibody. Antigen–antibody complexes were detected by the avidin–biotin peroxidase (ABC) method, using a commercially available kit (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) and visualized by 3,3'-diaminobenzidine (DAB, Sigma) in the presence of 0.2%  $(\text{NH}_4)_2\text{Ni}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  to enhance the immunostaining. Finally, the sections were counterstained with methylene blue/azure II in 1% sodium borate and distilled water. The sections were then dehydrated in graded alcohol series, cleared in xylene and coverslipped with Eukitt (O. Kindler, Freiburg, Germany). The sections were evaluated in a Nikon photomicroscope (Nikon Eclipse E600, Nikon Instruments Inc., Japan).

### Statistical analysis

All values are means  $\pm$  s.e.m. unless otherwise stated. The data were analysed with one-way analysis of variance (ANOVA) or one-way repeated measures analysis of

variance (RM-ANOVA) followed by Bonferroni's *t* test or Dunn's method (if the normality test failed). Student's paired *t* test was used for comparison of the COP measurements and unpaired *t* test for the distribution of fluid volumes.  $P < 0.05$  was considered statistically significant.

## Results

### Isolation of pulp fluid, validation of the method

In pilot studies, pulp tissue was exposed to consecutive centrifugations from 106 to 424 g and the extracellular tracer ratio (*R*) in pulp centrifugate and plasma was calculated. Centrifugation at 424 g for 10 min averaged a fluid to plasma ratio of  $^{51}\text{Cr-EDTA}$  tracer  $0.88 \pm 0.02$ , significantly different from 1. By modifications of the centrifugation procedure (change of G-force and centrifugation time) we found that centrifugation at 239 g for 10 min was optimal since the extracellular tracer ratio was  $1.05 \pm 0.05$  ( $n = 12$  rats), not significantly different from 1.0. The corresponding ratio for the intravascular marker  $^{125}\text{I-HSA}$  was  $0.08 \pm 0.01$  ( $n = 12$  rats).

The HPLC pattern of pulp fluid isolated by centrifugation is shown in Fig. 1B, which also shows the elution pattern of plasma (Fig. 1A), pulp eluate (Fig. 1C) and pulp homogenate (Fig. 1D) for comparison. The elution pattern of pulp fluid is quite similar to plasma except for a relatively large albumin fraction in the former. We compared the fluid isolated from pulp tissue by centrifugation with the fluid eluted into a buffer (pulp eluate) to see whether the centrifugation process *per se* affected the fluid composition. In addition, we isolated fluid from pulp tissue homogenate. The pulp eluate displayed an HPLC elution pattern similar to pulp centrifugate. The elution pattern of pulp homogenate resembled that of plasma and pulp centrifugate for proteins with hydrodynamic radius larger or similar to albumin, while this pattern differed for smaller molecules having several peaks in the area with lower molecular weight. This area constituted 0.1, 8.7, 11.6 and 13.8% of the total area under the UV220 elution curve of plasma, pulp centrifugate, eluate and homogenate, respectively.

### Fluid distribution volumes in pulp

Measurements of fluid distribution volumes in skin were performed in order to determine normal distribution of the isotopes at the extracellular and intravascular compartment. Total extracellular fluid volume ( $V_x$ ) averaged  $0.44 \pm 0.01$  ml (g w.w.) $^{-1}$  and vascular volume  $V_v = 0.005 \pm 0.0004$  ml (g w.w.) $^{-1}$ , in agreement with previous data (Gyenge *et al.* 2003). The incisor pulp exhibited a high content of IF compared to skin, as  $V_i$  averaged  $0.60 \pm 0.03$  ml (g w.w.) $^{-1}$  and vascular volume

$V_v = 0.03 \pm 0.01$  ml (g w.w.)<sup>-1</sup> (Table 1). No differences were observed in the fluid distribution volumes in the pulp after 1.5 and 3 h LPS exposure (Table 1).

### Pulpal blood flow and systemic blood pressure measurements during LPS exposure

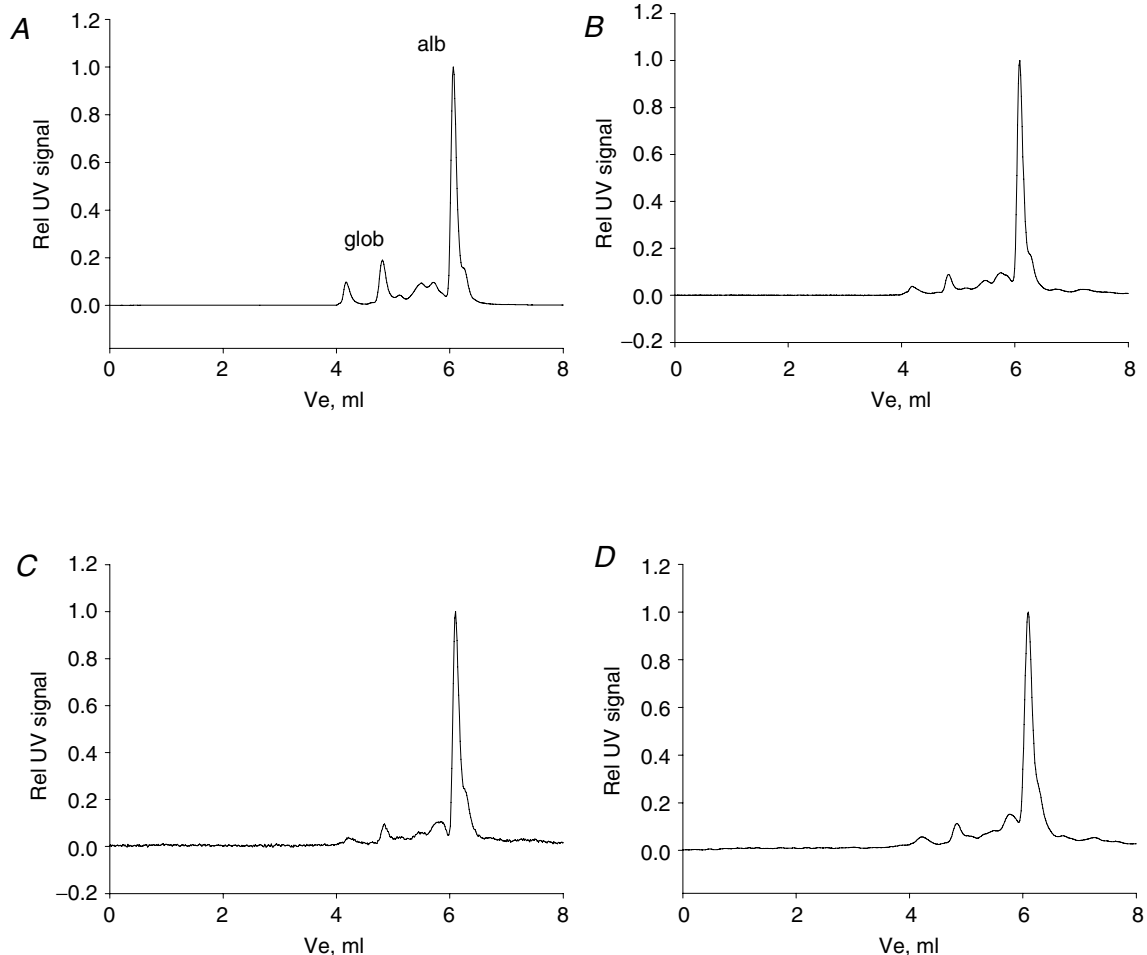
In 12 rats that received LPS, PBF was continuously recorded for 3 h. A significant drop in both PA and PBF within 10 min after LPS administration was observed. PA increased towards the end of the 3 h experimental period, whereas PBF remained low throughout the experimental period as exemplified in Fig. 2. PBF averaged  $26.0 \pm 4.8$  perfusion units (PU) before LPS administration with corresponding PA  $105.7 \pm 7.9$  mmHg ( $n = 12$ ) (Fig. 3). PA dropped significantly within 10 min after LPS administration to  $43.0 \pm 6.8$  mmHg

**Table 1. Interstitial fluid volume ( $V_i$ ) and vascular fluid volume ( $V_v$ ) in rat pulp after I.V. administration of LPS (4.0 mg kg<sup>-1</sup>) or vehicle (0.1% BSA in 0.9% NaCl)**

Group	<i>n</i>	$V_i$ (ml (g wet weight) <sup>-1</sup> )	$V_v$ (ml (g wet weight) <sup>-1</sup> )
Control	9	$0.60 \pm 0.03$	$0.03 \pm 0.01$
1.5 h LPS	6	$0.60 \pm 0.02$ (ns)	$0.03 \pm 0.002$ (ns)
3 h LPS	5	$0.55 \pm 0.05$ (ns)	$0.04 \pm 0.02$ (ns)

Values are means  $\pm$  s.e.m., *n* = number of rats, ns, not significant from control values, unpaired *t* test,  $P < 0.05$ .

( $P < 0.001$  compared to baseline values). PBF followed PA and dropped simultaneously to  $7.8 \pm 2.0$  PU after LPS administration ( $P < 0.001$ ) (Fig. 3). PA and PBF remained low for the first 1.5 h after LPS exposure with PA of  $76.5 \pm 8.2$  mmHg ( $P < 0.003$ ) and PBF of



**Figure 1. High performance liquid chromatography patterns**

Representative patterns for plasma and tissue fluid samples eluted in two TSK-gel size-exclusion columns coupled in series with an optimal separation range for globular proteins of 5–100 kDa and 10–500 kDa. The panels represent plasma (A), fluid isolated from the pulp of incisor teeth by centrifugation at 239 *g* for 10 min (B), pulp tissue eluate (C) and pulp tissue extract (D). alb, albumin; glob, globulins.

**Table 2. Cytokine levels (pg ml<sup>-1</sup>) in pulp interstitial fluid and serum from control rats (n = 8)**

	IL-1 $\alpha$	IL-1 $\beta$	IL-2	IL-6	TNF $\alpha$	IFN $\gamma$
Pulp IF	31.1 $\pm$ 16 (0–91)	52.7 $\pm$ 35.4 (0–229)	68.4 $\pm$ 45.5 (0–289)	217 $\pm$ 140 (0–760)	44.3 $\pm$ 23.9 (7.0–154)	56.9 $\pm$ 36.7 (0–199)
Serum	121.4 $\pm$ 109 (0–773)	2.0 $\pm$ 2.0 (0–14)	277.7 $\pm$ 201.7 (0–1440)	1643 $\pm$ 1513 (0–10705)	16.6 $\pm$ 7.3 (0–51)	513.7 $\pm$ 376.4 (0–2758)

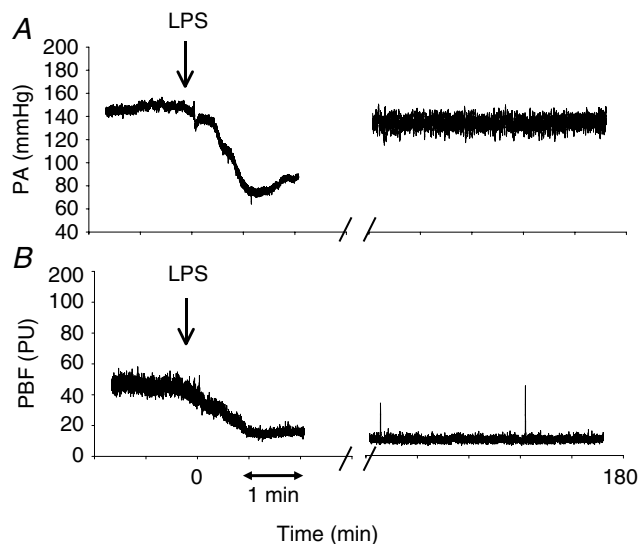
Values are means  $\pm$  s.e.m., range in parentheses.

11.2  $\pm$  2.2 PU ( $P < 0.001$ ) respectively (Fig. 3). At the end of the 3 h-observation period, PA increased to 84.0  $\pm$  11.9 mmHg, whereas PBF remained significantly lower (8.8  $\pm$  1.9 PU) than baseline values ( $P < 0.001$ ) (Fig. 3).

### Cytokine concentrations in serum and pulp IF

**Controls.** Serum and pulp IF from control animals exhibited undetectable or low levels of cytokines (Table 2). No differences were found in cytokine levels between serum and pulp IF (Fig. 4).

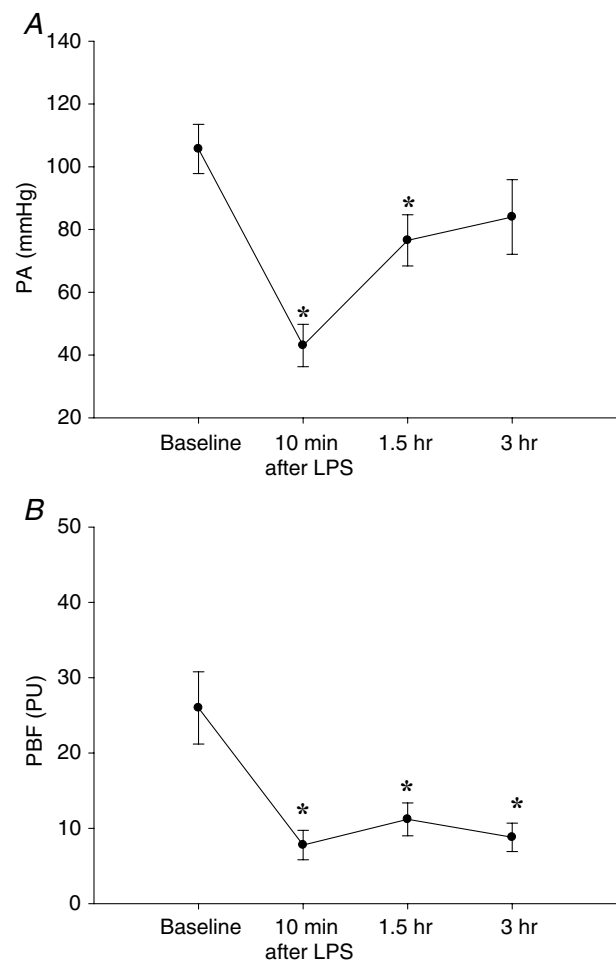
**LPS-treated animals.** The levels of the cytokines tested increased compared to control values in both serum and pulp IF, except for IL-2, which remained at constant levels during the experimental period (Fig. 4).



**Figure 2. Original simultaneous measurements of systemic blood pressure and pupal blood flow before, during and after lipopolysaccharide infusion**

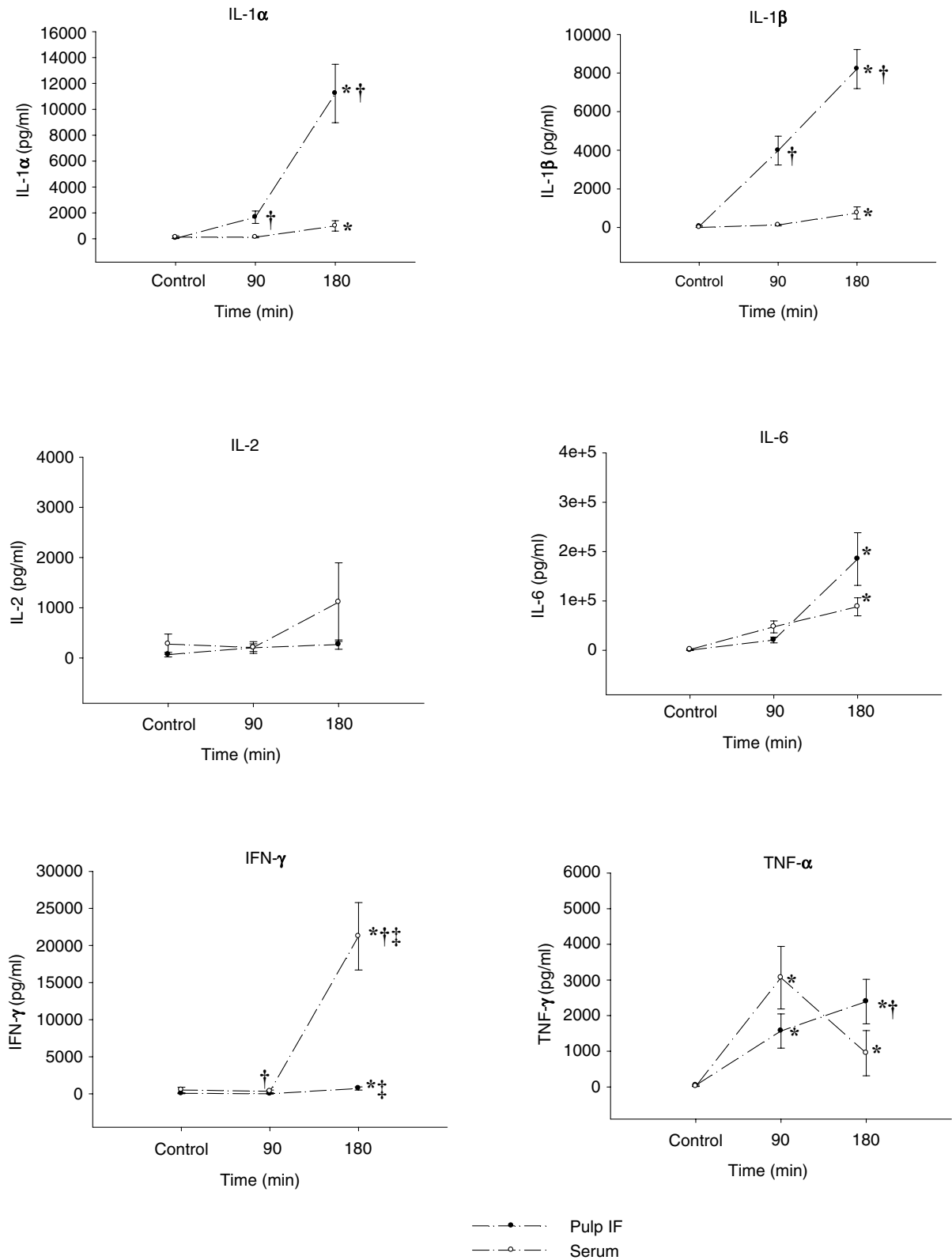
Original traces showing effect of intravenous injection of lipopolysaccharide (LPS), 4 mg kg<sup>-1</sup> resulting in an experimental endotoxaemia, on arterial systemic blood pressure (PA) (A) and pupal blood flow (PBF) (measured in arbitrary perfusion units (PU)) (B). Infusion of LPS (arrow) caused an almost immediate reduction in PA as well as PBF. Note that PA increased almost to levels seen before administration of LPS whereas, PBF remained low at the end of the 3 h-experimental period (right part of both panels).

IL-1 $\alpha$  level was statistically higher than control values in both serum (991  $\pm$  400 pg ml<sup>-1</sup>) and IF (11222  $\pm$  2255 pg ml<sup>-1</sup>) after 3 h. Local production of IL-1 $\alpha$  in the pulp was evident as a several-fold higher concentration of this cytokine was found in



**Figure 3. Effect of lipopolysaccharide on blood pressure and pupal blood flow**

Effect of intravenous injection of lipopolysaccharide (LPS), 4 mg kg<sup>-1</sup>, resulting in an experimental endotoxaemia, on arterial systemic blood pressure (PA) (A) and pupal blood flow (PBF) (B) under control conditions (baseline) and various time points after infusion of LPS. PBF measured in arbitrary perfusion units (PU). Values are means  $\pm$  s.e.m.,  $n = 12$ ; one-way RM-ANOVA, \* $P < 0.05$  compared with baseline measurements.



**Figure 4. Effect of lipopolysaccharide on cytokines in pulp interstitial fluid and serum**

Concentration of cytokines in pulp interstitial fluid and serum in experimental endotoxaemia induced by intravenous injection of lipopolysaccharide (LPS), 4 mg kg<sup>-1</sup>. The experiment ended 90 min (n = 6) and 180 min (n = 6) after LPS administration. Controls (n = 8) received vehicle alone and were kept for an equivalent time (90 min, n = 4 and 180 min, n = 4). Values are means  $\pm$  S.E.M.; ANOVA, \*P < 0.05 compared with respective control values, †P < 0.05 compared with serum at the same time interval, ‡P < 0.05 compared with respective values at 90 min.

pulp IF compared to serum after 1.5 and 3 h (Fig. 4). The same pattern was found for IL-1 $\beta$  with increased levels seen 3 h after LPS administration ( $748 \pm 318$  and  $8211 \pm 1013$  pg ml $^{-1}$  for serum and pulp IF, respectively) when compared with controls. Again, pulp IF expressed higher levels of IL-1 $\beta$  than serum throughout the observation period, indicating local production of this cytokine in the pulp tissue (Fig. 4).

IL-6 was increased at 3 h ( $88194 \pm 18401$  and  $184958 \pm 53275$  pg ml $^{-1}$  for serum and pulp IF, respectively) compared with controls. No difference in IL-6 concentration was observed between serum and IF (Fig. 4).

IFN- $\gamma$  increased during the experimental period and 3 h post-LPS treatment. The concentration of IFN- $\gamma$  was higher in both serum ( $21242 \pm 4547$  pg ml $^{-1}$ ) and IF ( $734 \pm 219$  pg ml $^{-1}$ ) compared with control values and levels exhibited at 1.5 h ( $336 \pm 173$  and  $10 \pm 6.4$  pg ml $^{-1}$  for serum and pulp IF, respectively). There was a differential response in serum and IF as well, but this inflammatory mediator showed higher levels in serum than in IF at both 90 and 180 min (Fig. 4).

TNF- $\alpha$  was also found to be increasing throughout the observation period with a slightly different pattern: the levels of TNF- $\alpha$  were significantly higher at 90 min ( $3061 \pm 878$  and  $1569 \pm 481$  pg ml $^{-1}$  for serum and pulp IF, respectively) and 180 min ( $946 \pm 635$  and  $2393 \pm 623$  pg ml $^{-1}$  for serum and pulp IF, respectively)

compared with control values for both serum and IF, but it seemed that serum concentration reached a peak at 90 min, whereas TNF- $\alpha$  in IF continued to rise. There was a difference in TNF- $\alpha$  concentration between serum and pulp IF at 180 min (Fig. 4).

### COP measurements

COP in pulp IF of control rats averaged  $19.6 \pm 1.3$  mmHg with a corresponding COP in plasma of  $23.6 \pm 0.8$  mmHg ( $n = 7$ ) giving a significant difference ( $P < 0.05$ ). The pulp fluid COP corresponded to 83% of control plasma COP. Since most differences in cytokine levels were found 3 h after LPS challenge, we measured COP in pulp fluid and plasma only in rats exposed to LPS for 3 h ( $n = 8$ ). In three of these rats, plasma COP at the beginning of the experiments averaged  $19.3 \pm 0.3$  mmHg. Three hours after LPS exposure, COP in pulp IF averaged  $15.6 \pm 1.1$  mmHg ( $80.5 \pm 5.6\%$  of control plasma COP ( $n = 3$ )) whereas plasma COP dropped to  $14.8 \pm 1.0$  mmHg ( $76.3 \pm 5.4\%$  of control plasma COP). There was no difference in COP levels between pulp IF and plasma at 3 h after LPS exposure.

### Immunostaining

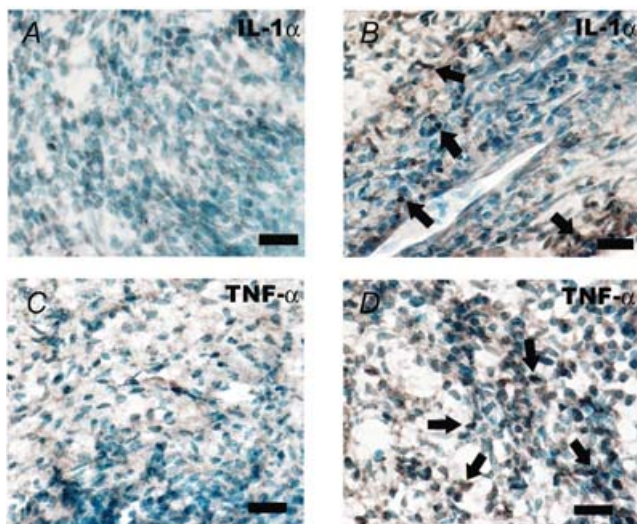
Since local production of IL-1 $\alpha$  and TNF- $\alpha$  was found in LPS exposed pulps, we aimed at verifying the results by immunohistochemical staining of the pulp for the above mentioned cytokines.

Pulp sections from the control rat exhibited scarce IL-1 $\alpha$ -immunoreactive (IR) cells and TNF- $\alpha$ -IR cells in the pulp body. Pulp tissue from the rat exposed to LPS showed an increased number of both IL-1 $\alpha$ - and TNF- $\alpha$ -IR cells in the pulp proper. The stained cells had either a round or an elongated appearance indicative of immune cells or fibroblasts, respectively (Fig. 5).

All the negative controls showed a lack of specific immunostaining.

### Discussion

The microenvironment of the pulpal cells has not been thoroughly explored. One reason for this is that the nature of tooth pulp does not permit direct access without harming the tissue. Dentinal fluid represents pulp IF collected from cut dentin surfaces. Data regarding its composition has been published (Haldi *et al.* 1965; Coffey *et al.* 1970; Knutsson *et al.* 1994) but cutting the dentin surface and exposing the dentinal tubules induces inflammatory reactions in the tissue. In this study, we showed that it is possible to obtain pulp IF by centrifugation of pulp tissue at low *G*-force. By isolation of the pulp after cardiac arrest, we avoided inflammatory changes in the IF composition due to the procedure, and



**Figure 5. Immunohistochemical microphotographs of dental pulp from untreated and lipopolysaccharide-exposed rats**  
 A, saggital section of incisor pulp from control rat showing no IL-1 $\alpha$ -immunoreactive (IR) cells in the pulp body. B, pulp section of rat challenged with lipopolysaccharide (3 h) showing numerous IL-1 $\alpha$ -IR cells (arrows). Note that the stained cells are either round or elongated. C, few TNF- $\alpha$ -IR cells in the pulp tissue proper of control rat, whereas increased number of TNF- $\alpha$ -IR cells appears 3 h after LPS exposure (D) (arrows). Bars, 10  $\mu$ m.



we could measure the levels of inflammatory mediators, as well as COP in the pulp IF. New information concerning intercellular communication and fluid exchange during health and disease could thereby be provided.

The pulp houses a number of tissue elements, namely nerves, vascular tissue, connective tissue fibres, ground substance, IF and cellular components. Cell compression during centrifugation may lead to the extrusion of cellular fluid, resulting in the isolation of a mixture of IF and cell fluid.  $^{51}\text{Cr}$ -EDTA was used as an extracellular marker. This probe is not metabolized and is not taken up by cells. A reduced ratio ( $R$ ) of  $^{51}\text{Cr}$ -EDTA concentration (centrifugate relative to plasma) could reveal possible 'contamination' with cellular fluid. We evaluated the isolated fluid and concluded that centrifugation of the pulp tissue at 239 g for 10 min was optimal for the isolation of fluid representative for pulp IF since  $R$  was not significantly different from 1.0. Increased time and/or G force for centrifugation showed a reduction in  $R$ , suggesting dilution of the pulp centrifugate with intracellular fluid. The centrifugate was found to contain 8% of the intravascular tracer ( $^{125}\text{I}$ -HSA). In addition, the HPLC pattern of isolated pulp fluid was similar to that of the plasma, and not different from the pattern of pulp tissue eluate (Fig. 1), indicating that the centrifugation process *per se* did not cause serious cell damage. The impression of a lower share of globulins in isolated pulp fluid than in plasma most likely reflects a size selectivity of the capillary membrane. Also the HPLC pattern of the pulp homogenate was similar to that of the pulp fluid. Only a small amount of low molecular weight substances were eluted in the column after albumin, most likely representing intracellular proteins. The dental pulp has a very high total water content (75% of total weight) (Berkovitz *et al.* 2002) with 63% localized extracellularly (present study), showing relatively low intracellular fluid volume (12%). It seems therefore reasonable to assume that the somewhat unexpectedly small contribution of non-plasma proteins of pulp tissue homogenate can be explained by its relatively low content of cellular components. Taken together, our data suggest that low speed pulp centrifugation is indeed a simple and reliable method for the isolation of pulp IF.

We showed that in normal pulp, the fluid isolated by centrifugation had a COP of  $\sim 83\%$  of that in plasma, showing a relatively high protein concentration in the pulp fluid during physiological condition, i.e. significantly higher than COP found in skin (Wiig *et al.* 2003) and muscle (Wiig *et al.* 1991). Three hours after LPS exposure, COP had fallen in plasma suggesting a substantial protein leakage to the interstitium of other tissues and areas (intestines, abdomen) (Aust *et al.* 1957; Dormehl *et al.* 1992) whereas the relative pulp fluid COP remained unchanged (80.5% of control plasma COP). It is likely that the permeability of the pulp vessels was increased after the LPS challenge as the COP in pulp IF and plasma was similar

after the exposure. Furthermore, cytokines known to cause increased permeability, such as IL-1 (Martin *et al.* 1988; Daffonchio *et al.* 2002), were up-regulated and LPS as such is known to have a direct effect on the endothelial barrier and to cause increased vascular permeability (Bannerman & Goldblum, 1999, 2003). In addition, the reduced pulp perfusion after LPS exposure most likely resulted in a reduced capillary pressure favouring fluid reabsorption from the interstitium and thereby an increased interstitial fluid colloid osmotic pressure. Such a mechanism may also partly explain our finding of a similar COP in plasma and pulp fluid in this situation. The fact that we found no changes in fluid volumes in the pulp during this model of acute inflammation may indicate that the dental pulp, enclosed in hard dentin, is such a low-compliant tissue that significant changes in fluid volumes are not possible or are too small to be detected with the method used.

However, PBF was significantly lower 3 h after LPS challenge, even though the systemic arterial pressure normalized, indicating impaired pulpal circulation. Organ failure occurs commonly in sepsis even after restoration of haemodynamic status, and can be related to the direct impairment of cellular function or hypoxia and/or redistribution of blood flow between and within the organs at the microcirculatory level (King *et al.* 1999; De Backer *et al.* 2002). De Backer *et al.* (2002) reported a reduced number of continuously perfused small vessels ( $< 20 \mu\text{m}$ ) in the sublingual area in septic patients compared with controls. The very low perfusion observed in the pulp after LPS application, even after restoration of the systemic blood pressure, indicates that the pulpal tissue was seriously affected by LPS and developed organ dysfunction. In the clinical situation, bacterial infection frequently induces pulpal necrosis after periods with insufficient perfusion. The vulnerable pulp, due to its lack of collateral circulation, is prone to necrotize, resulting in the need for root canal treatment.

To the best of our knowledge, this is the first report quantifying cytokines in IF of normal and inflamed pulps. The IF has traditionally been considered a transport medium for nutrients and waste products, but it also represents a communication medium between cells. There are several reports regarding elevated levels of cytokines in pulpal inflammation (Tani-Ishii *et al.* 1995; Rauschenberger *et al.* 1997; Barkhordar *et al.* 1999; Kim & Lim, 2002; Pezelj-Ribaric *et al.* 2002; Zehnder *et al.* 2003), but these were done in tissue homogenates including both cellular and blood-derived cytokines in the samples. In addition, measurements of cytokines in both serum and IF could give more information on inflammatory processes occurring locally at tissue level.

We investigated six of the most common pro-inflammatory cytokines and a main finding was the differential cytokine response in serum and pulp IF. Low levels of cytokines were detected in the control rats in

agreement with previously reported data from normal pulp (Bletsa *et al.* 2004; Kawashima *et al.* 2005). Elevated levels of cytokines were found after LPS challenge but the main finding was the differential cytokine response in serum and pulp IF. Specifically, IL-1 $\alpha$  and IL-1 $\beta$  showed up to 11 times higher concentrations in pulp IF compared with serum after 3 h LPS challenge. This indicates that these cytokines are locally produced in the pulp and released into the interstitium. This finding may be explained by the abundance of various types of macrophages in the normal dental pulp (Jontell *et al.* 1987; Okiji *et al.* 1992) and their role in defence mechanisms following exogenous invasion of pathogenic stimuli. Activated macrophages represent the major source of IL-1, and are likely to be responsible for the high local production of the pro-inflammatory cytokines IL-1 $\alpha$  and IL-1 $\beta$  in inflamed pulp since the producing cells already reside in the tissue. In addition, cells at the odontoblastic layer of normal rat pulp stain positively for IL-1 $\alpha$  (Bletsa *et al.* 2004) and they might play a role in this prompt cytokine production during pulpitis. Immunohistochemical analysis of pulp tissue confirmed the presence of numerous IL-1 $\alpha$ -IR cells 3 h after LPS exposure.

IFN- $\gamma$  concentration in the serum was significantly higher than pulp IF, suggesting cytokine transport across the microvasculature from plasma to pulp IF. Although local production of this mediator cannot be excluded, it is more reasonable to conclude that it is blood derived since it was the only cytokine found at higher concentration in serum compared to pulp IF throughout the whole experimental period. Natural killer cells (NK) are the predominant IFN- $\gamma$ -producing cells after LPS challenge (Varma *et al.* 2002) and they are scarcely found in normal pulp. The diverse source of IFN- $\gamma$  compared to the locally produced IL-1 could clarify the difference in levels of those cytokines in serum and IF.

A surprising result was that there were minimal changes in the IL-2 levels after endotoxin administration. There are contradictory results in the literature regarding IL-2 levels in dental pulps. Elevated levels of IL-2 have been related to pulpal inflammation (Rauschenberger *et al.* 1997). However, later studies found no differences in IL-2 levels in healthy and diseased pulps, suggesting a favourable Th2 cytokine production in inflamed pulp tissue (Anderson *et al.* 2002). A shift in cell responses from Th1 to Th2 subtype appears to be a common finding in sepsis, with the ultimate goal of protecting the organism from systemic 'overshooting' with Th1 pro-inflammatory cytokines (Ayala *et al.* 1994) and IL-2 belongs to the latter category of cytokines. It should be kept in mind that this study utilized a model of sepsis in order to achieve pulpitis.

IL-6 was the cytokine that expressed the highest levels in both IF and serum. No differences were seen between pulp IF and serum. It cannot therefore be concluded

that this cytokine is systemically and/or locally produced under the experimental conditions. IL-6 represents the main mediator of the acute phase reaction that takes place shortly after the onset of infections (Akira *et al.* 1990; Kishimoto *et al.* 1992), and it and IL-1 and TNF- $\alpha$  are the major pro-inflammatory cytokines involved in the septic condition (Ertel *et al.* 1991). Physiological stimuli for its synthesis are LPS, IL-1 and TNF- $\alpha$ , all present in high levels in the current model of acute inflammation. Production of IL-6 after bacterial challenge of cultured human pulp cells (Matsushima *et al.* 1998; Tokuda *et al.* 2001), as well as detection of increased levels of IL-6 in inflamed human pulp and periapical lesions (Barkhordar *et al.* 1999), strongly suggests the involvement of this cytokine in pulpal inflammation.

TNF- $\alpha$  reached a peak in serum at 90 min after LPS challenge. By contrast, TNF- $\alpha$  continued to rise in the pulp IF throughout the 3 h experimental period. TNF- $\alpha$  is secreted by macrophages, monocytes, neutrophils, T-cells and NK cells following stimulation by bacterial LPS, justifying both local and systemic production of this cytokine. Locally, in the pulp, up-regulation of TNF- $\alpha$ -IR cells was observed 3 h after LPS challenge.

In a recent study exploring the role of cytokines in pulpal and periapical inflammation, the kinetics of pro-inflammatory cytokine expression were evaluated in a rat pulpitis model (Kawashima *et al.* 2005). Slight expression of IL-1 $\alpha$  and IL-1 $\beta$  was observed at the onset of the experiment. LPS applied directly in contact with the pulp tissue after pulp exposure induced the expression of IL-6 and TNF- $\alpha$  and also enhanced the expression of IL-1 $\alpha$  and IL-1 $\beta$  at 3 h with a peak for all the above cytokines at 6 h. Although a different quantification method was used (RT-PCR) on the entire pulp tissue extract, our results can be related to this study since IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$  and IL-6 had significantly higher levels in the pulp IF at 3 h post LPS-treatment.

Few studies exist on cytokines in IF but recently, levels of IL-1 $\beta$  and TNF- $\alpha$  in skin IF and serum from rats with endotoxaemia were measured (Nedrebø *et al.* 2004) and a differential cytokine response in serum and skin IF was shown, with local production of IL-1 $\beta$  and systemic production of TNF- $\alpha$ . Our results are in line with the results from skin regarding local production of IL-1. However, also TNF- $\alpha$  was found to be locally produced in the pulp, suggesting a different tissue response to LPS challenge between skin and dental pulp. IL-1 and TNF- $\alpha$  have been shown to induce lowering of interstitial fluid pressure thereby facilitating oedema in skin and oral mucosa (Nedrebø *et al.* 1999; Bletsa *et al.* 2006). We can only speculate about the detrimental effect of this local IL-1 and TNF- $\alpha$  production during inflammation in the pulp that has extremely limited ability to expand.

There is growing evidence that cytokines are involved in the generation of pain and hyperalgesia (Sommer

& Kress, 2004. The pathways by which cytokines affect brain function have also been under investigation. Cytokine-to-brain communication via blood is one route, but it cannot explain the fact that illness responses are often observed under situations in which blood levels of cytokines are very low (Kluger, 1991). Therefore a neural route rather than a humoral one has been suggested (Watkins *et al.* 1995). By such a mechanism, cytokines do not need to rise systemically because increases in local tissue concentrations would be sufficient to induce illness responses and pain. In this context, our findings of local production of IL-1 and TNF- $\alpha$  in the nerve-rich dental pulp may have a direct clinical significance in pain development during pulpitis.

In summary, this study established a reliable method for isolating pulp IF. We applied this method for measuring cytokine levels and colloid osmotic pressure in pulp IF in order to study the pathophysiology of pulpitis. We have shown that certain pro-inflammatory mediators were markedly up-regulated in the pulp interstitium during acute pulpitis. Moreover, we obtained novel data regarding fluid exchange in dental pulp during health and disease. Analysis of pulp IF offers a better understanding of the mechanisms involved in inflammatory reactions.

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