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Alterations in liver parenchyma after sialoadenectomy in mice: Contribution of neutrophils and macrophages to the removal of damaged hepatocytes

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Summary. Surgical excision of submandibular salivary glands (sialoadenectomy) alters cell turnover in mice liver. Here we show that the liver of adult mice contained scattered leukocyte infiltration foci whose size was in the range of the diameter of hepatocytes. The number of infiltration foci in the liver increased soon after sialoadenectomy and remained high for several weeks. Neutrophils were recruited on dying hepatocytes soon after the initiation of the apoptotic process. Kupffer cells appeared later in the process. Just 2 days after sialoadenectomy, the number of type I infiltration foci (corresponding to the first stage) had increased 5-fold. Since these alterations in liver structure are coincident with a transient decrease in plasma EGF concentration, we studied whether inhibition of EGF receptor by means of genistein injection produced a similar effect. After three days of genistein administration, the number of type I infiltration foci increased 3.5-fold. Sialoadenectomized mice were more susceptible than controls to endotoxin shock. While 90% of sham-operated mice survived a burst of 100 µg lipopolysaccharide/Kg (combined with D-galactosamine 750 mg/Kg), only 50% of sialoadenectomized mice survived. The surviving sialoadenectomized mice recovered more slowly than the controls, as indicated by the high plasma alanine transaminase activity a week after the burst. We conclude that (i) neutrophils and macrophages participate in the process of apoptotic hepatocyte removal in a sequential manner; (ii) although the alteration of liver structure induced by sialoadenectomy is mild, it has delayed consequences on the ability of the liver to deal with aggressive insults.

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Introduction

Submandibular salivary glands accumulate a number of biologically active peptides that are released into both saliva and the bloodstream (Boyer et al., 1991; Rougeot et al., 2000). One of these peptides is Epidermal Growth Factor (EGF). In male mice, EGF represents nearly 0.5% of the total gland protein (Grau et al., 1994).

The liver is a major target organ for EGF. Hepatocytes contain the largest number of EGF receptors (known as ErbB1) (O'Keefe et al., 1974; Soley and Hollenberg, 1987) and the existence of a submandibular gland - liver axis has been suggested (Marti et al., 1989). The basis for this axis is that the liver is very efficient at extracting EGF from the portal vein (Hilaire et al., 1983; Jansen et al., 2003). We estimated that the liver accounts for nearly 70% of the whole animal clearance of EGF released from submandibular glands after adrenergic stimulation (Grau et al., 1994). Part of the EGF taken up by the liver ends up in bile (Hilaire et al., 1983), where it reaches concentrations that are 100-fold higher than in plasma (Grau et al., 1994). It has been suggested that a plasma-liver-bile route delivers EGF to the upper small intestine, where it may regulate several processes including pancreatic secretion (Jansen et al., 2003).

EGF not only passes through the liver, but also has an important role in this organ. It is a potent mitogen for hepatocytes (McGowan et al., 1981). Thus, in cultured cells EGF has been shown to make a relevant contribution to liver regeneration after partial hepatectomy (Michalopoulos and Defrances, 1997). EGF affects liver metabolism in the direction that

supports the mitogenic effect (Conricode and Ochs, 1990; Peak and Agius, 1994; Gómez-Lechón et al., 1995; Quintana et al., 1995; Grau et al., 1996). In recent years, a novel function of EGF has been identified. It protects the liver against a variety of insults, such as endotoxins (Cho et al., 2000; Deaciuc et al., 2002), drugs (acetaminophen) (Grunnet et al., 2003), and apoptotic agents (Roberts et al., 2000; Ethier et al., 2003; Musallam et al., 2004).

In mice, sialoadenectomy has been shown to be a good tool for studying EGF function in whole animals (Noguchi et al., 1991; Grau et al., 1997; Fernández et al., 2000; Tebar et al., 2000). We observed that shortly after surgery, the EGF concentration in plasma decreased to undetectable levels. It then slowly recovered (Buira et al., 2004). This is coincident with a transient increase in apoptosis, as determined by in situ TUNEL labeling. The increase in apoptosis is followed by an increase in DNA synthesis, but not cell division. This leads to a reduction in the number of remaining hepatocytes accompanied by an increase in their size (Buira et al., 2004).

The morphological changes in apoptotic hepatocytes have been studied in detail (Feldmann 1997). The involvement of neighboring cells, including Kupffer cells, in the phagocytosis of apoptotic bodies has been clearly demonstrated (Dini et al., 1995, 2002; Canbay et al., 2003). Even though neutrophil recruitment occurs early after the induction of apoptosis (Lawson et al., 1998), it is usually associated with an extension of tissue injury and oncotic necrosis (Jaeschke, 2006).

Although TUNEL labeling is associated with apoptosis, necrotic processes may also render TUNEL-positive hepatocytes (Grasl-Kraupp et al., 1995). Here, we studied the contribution of neutrophils and macrophages to the removal of dying hepatocytes. We found that the recruitment of neutrophils onto damaged hepatocytes is an early event that precedes macrophages. We did not find evidence of progression to necrosis in the liver of sialoadenectomized mice.

Material and methods

Animals

Adult Swiss-CD1 mice were obtained from Interfauna (Barcelona, Spain). All animals were male (35-40 g), fed *ad libitum*, and kept under a constant 12-h light-dark cycle (lights on at 8:00) in controlled humidity (45-55%) and temperature (25±1°C) conditions. All experimental procedures were approved by the Committee on Animal Care of the University of Barcelona and by the Conselleria de Ramaderia i Pesca, Generalitat de Catalunya.

Sialoadenectomy

Sialoadenectomy was performed as described elsewhere (Grau et al., 1994). Briefly, a small incision was made in ketamine/xylacine anaesthetized mice to expose the submandibular salivary glands, which were then ligated and excised. In control (sham-operated) animals, the glands were exposed and a ligature was passed, but not tied. The wound was stitched and disinfected. Sham-operated and sialoadenectomized animals were maintained in fasting conditions for the next 24h. Thereafter, animals had free access to pelleted chow and tap water until they were killed.

Endotoxin treatment

In some experiments, animals received lipopolysaccharide (LPS, 100 µg/Kg i.p.) from *Salmonella abortus equi* (Sigma-España, Madrid, Spain) combined with D-(+)-galactosamine (Calbiochem, Merck, Barcelona, Spain) 750 mg/Kg (i.p.) dissolved in phosphate-buffered saline. At the indicated times, blood (0.1 mL) was obtained from the tail to determine alanine aminotransferase (see below).

Genistein treatment

In some experiments, mice were injected (s.c.) with the EGF receptor (ErbB1) inhibitor genistein (Akiyama et al., 1987) at the dose of 5 mg/mouse (Sigma-España, Madrid, Spain) twice a day during 3 consecutive days. Control mice were injected with the vehicle (0.05 mL ricine oil). Twelve hours after the last injection, mice were sacrificed. In a preliminary experiment, mice were injected with a single dose of 0 or 1 or 5 mg genistein. Twelve hours later they received 2.5 µg EGF (i.p., dissolved in sterile PBS; Roche Diagnostics, Sant Cugat del Vallès, Barcelona, Spain) and sacrificed after 10 min.

Sampling and analysis

To obtain samples, mice were anaesthetized (sodium pentobarbital 90 mg/Kg). Blood was collected from the inferior vena cava into heparinized syringes. Blood plasma was obtained by centrifugation. Aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase activities in plasma were determined as previously described (Sánchez et al., 2002). Plasma cytokines were quantified by ELISA using commercial kits (Biosource International). Immediately after bleeding, the liver was perfused in situ with phosphate-buffered saline supplemented with heparin (1.25 mg/mL). Then, an approximately 500 mg portion was fixed in 4% paraformaldehyde and embedded in paraffin to obtain 7 µm-thick sections for hematoxylin-eosin (HE) staining or immunohistochemistry. In some experiments, the rest of the liver was stored at -80°C until it was used to determine myeloperoxidase (MPO) activity as indicated in (Buira et al., 2004).

Macrophage (Kupffer cell) staining in liver sections

Seven-µm liver sections were collected on glass slides coated with gelatin. After deparaffination and

hydration, the slides were rinsed in PBS (3x5 min). To inactivate endogenous peroxidase, the slides were incubated (10 min) at room temperature with 0.5% H_2O_2 in PBS containing 5% triton X-100 and 10%methanol. After rinsing (4x5 min in PBS and 5 min in PBS containing 0.2% triton X-100), nonspecific binding sites were blocked for 2h at room temperature in a humidified chamber, with 10% goat serum (Gibco-BRL, Invitrogen, El Prat de Llobregat, Spain) in PBS containing 0.5% triton X-100, 0.2 M glycine and 0.2% gelatin. Slides were rinsed briefly in 0.1 M Tris-HCl pH 7.6 and incubated overnight at 4°C in a humidified chamber with the primary antibody (rat anti-mouse F4/80: AbD-Serotec, Bionova, Barcelona, Spain) diluted 1/50 in PBS containing 10% goat serum, 0.5% triton X-100, 0.2% gelatin and 0.02% sodium azide. After rinsing (2x5 min in 0.2% triton X-100 in PBS), sections were incubated for 2 h at room temperature in a humidified chamber with the secondary antibody (goat anti-rat IgG conjugated with horseradish peroxidase, mouse adsorbed: AbD-Serotec, Bionova, Barcelona, Spain) diluted 1/25 in PBS containing 10% goat serum, 3% Triton X-100 and 0.2% gelatin. Finally, slides were rinsed (5 min in PBS, 2x5 min in 0.2%Triton X-100 in PBS, and 2x10 min in 0.1 M tris-HCl pH 7.6) and developed (12 min incubation in a solution containing 0.003% H_2O_2, 0.025% 3',3'-diamonobenzidine and 0.17% nickel-ammonium sulfate). The reaction was stopped with 0.1 M Tris-HCl pH 7.6 and slides were immediately counterstained with methyl green. After a brief dehydration process, the slides were mounted in DPX. Sections were analyzed in a Zeiss Axiostar-Plus microscope.

Neutrophil staining in liver sections

Seven-µm liver sections were collected on glass slides coated with poly-L-lysine. After deparaffination and hydration, the slides were incubated (2x30 min) in 0.34% $NaBH_4$ in PBS to reduce basal fluorescence and rinsed in PBS (3x5 min). Liver sections were permeabilized (10 min at room temperature in trypsin-EDTA solution, Gibco-BRL, Invitrogen, El Prat de Llobregat, Spain), rinsed (2x5 min in PBS and 5 min in 0.2% Triton X-100 in PBS), and nonspecific binding sites were blocked for 2h at room temperature in a humidified chamber with 10% goat serum in PBS containing 0.52 M NH_4Cl, 0.5% triton X-100, 0.2 M glycine and 5% defatted milk powder. Slides were rinsed briefly in 0.1 M Tris-HCl pH 7.6 and incubated for 90 min at room temperature in a humidified chamber with the primary antibody (rat anti-mouse neutrophil antigen 7/4: AbD-Serotec, Bionova, Barcelona, Spain) diluted 1/300 in PBS containing 10% goat serum, 0.5% Triton X-100, 5% defatted milk powder and 0.02% sodium azide. After rinsing (2x5 min in 0.2% triton X-100 in PBS), sections were incubated for 90 min at room temperature in a humidified chamber with the secondary antibody (Alexa Fluor 594 goat anti-mouse IgG: Molecular Probes, Invitrogen, El Prat de Llobregat, Spain) diluted 1/300 in PBS containing 10% goat serum, 3% Triton X-100 and 3% defatted milk powder. After rinsing (5 min in PBS), sections were mounted in Mowiol (Fluka-Sigma-Aldrich, Madrid, Spain) and immediately analyzed in a Leica DM IRB fluorescence microscope.

Histopathological score

In some experiments the effect of endotoxin inducing liver injury was scored on liver sections. Transverse sections (7 µm thick) of paraffin-embedded livers were stained with hematoxylin-eosin. The morphological changes were scored as follows: 1, normal appearance; 2, focal or diffuse leukocyte infiltration; 3, small group of damaged hepatocytes (affected area < 1/4); and 4, large necrotic area (> 1/3) with or without hemorrhage. Twenty high-powered fields (HPF: x400 magnification) per slice were scored and 4 randomly selected slices per animal were analyzed.

Perfusion of isolated livers

Mice were injected (i.p.) with heparin solution (0.1 mL, 2.5 mg/mL) at 10 min before anaesthesia with sodium pentobarbital 90 mg/Kg (i.p.). The inferior vena cava was cannulated and the liver was retrogradely perfused (2 mL/min) for 10 min with Leibovitz solution (Leibovitz 1963) supplemented with 20 mM Hepes, 15 mM glucose, 1.3 mM $CaCl_2$ and vitamins and amino acids as in (Galan et al., 2002) and gassed with O_2/CO_2 (95/5%). During this time, the liver was carefully isolated and placed in a perfusion chamber maintained at 37°C. The perfusion continued for 3 h in non-recirculation conditions. At indicated times, a sample of the perfusate was collected. Samples were immediately brought to 3% albumin and kept at -20°C until used for cytokine and lactate dehydrogenase quantification (see above).

Western blot analysis of ErbB1 and phospho-Tyr containing proteins

Liver samples were homogenized and processed for polyacrylamide-gel electrophoresis and Western blotting as in (Pareja et al., 2003). In ErbB1 western blots, the primary antibody was obtained from Santa Cruz Biotech. (cat n. EGFR (1005): sc-03) (Quimigranel, San Fernando de Henares, Madrid, Spain) and it was used at 1/1.000 dilution. The secondary antibody (peroxidase-conjugated GaR-IgG) was provided by Jackson ImmunoResearch (cat n. (111-035-008) (S.G. Servicios Hospitalarios, Barcelona, Spain) and used at 1/20.000 dilution. In phospho-Tyr western blots, the primary antibody (anti-phosphotyrosine, clone PY20) was obtained from Upstate (cat n. 05-947) and was used at 1/2.000 dilution. The secondary antibody (peroxidase-

conjugated GaM-IgG) was provided by Jackson ImmunoResearch (cat n. (115-035-008) and was used at 1/5.000 dilution.

Statistical analysis

All results are the mean ± S.E.M. of the number of animals indicated in each figure. The statistical significance of the differences was determined by one-way or two-way ANOVA and post-hoc Tukey's test, depending on the experimental design. Survival curves were analyzed by Kaplan-Meier and the differences tested by the log Rank test. Histopathological scores were compared by the non-parametric Mann-Witney test.

Results

HE stained liver sections from control or sialoadenectomized mice showed focal points of infiltrated cells (arrowhead in Fig. 1A). These points were not distributed regularly. Instead, they were more frequently observed near lobe edges. To better understand the nature of this focal accumulation, we analyzed consecutive sections of 5 liver samples. We observed that infiltration focal points always extended between 4 and 8 consecutive sections. This means that such points extend through 30-55 µm, which is in the range of a single hepatocyte diameter. Figs. 1B to 1G show a representative focal point.

If we consider that each infiltration focal point corresponds to a hepatocyte in some stage of the death process, the number of such focal points could be used to estimate the number of dying hepatocytes. Of course, this is an underestimation because it does not take into consideration dying cells that have not recruited leukocytes. Nevertheless, since we have shown (Buira et al., 2004) that sialoadenectomy results in a transient increase in TUNEL-labeled cells in the liver, we counted the number of focal infiltration points in the liver of control and sialoadenectomized mice (Fig. 2). Sham-operated mice maintained a constant number of dying hepatocytes: 10 to 15 cells per 100 low-powered fields (LPF). Since we estimated that there is a mean of 9,000 hepatocytes/LPF, one out of 60,000-90,000 hepatocytes underwent a death process in control mice. One week after sialoadenectomy the number of dying hepatocytes increased 3-fold. It remained high until the third week after surgery and decreased thereafter.

The observation of nearly 2,500 leukocyte infiltration foci revealed that their morphology was not homogeneous. We identified three distinct types. The first appeared typically as a single hepatocyte with pyknotic nuclei (sometimes karyorrhexis was observed instead), surrounded by infiltrated cells (Fig. 3A). The second type was a dense aggregate of infiltrated cells. No hepatocytes were visible in this type (Fig. 3B). The third type appeared as a less dense aggregate with a void space in between cells (Fig. 3C).

Although recruitment of neutrophils appears to be an early process in apoptosis (Lawson et al., 1998), their contribution to the clearance of apoptotic cells is not always recognized (Feldmann, 1997; Dini et al., 2002). Therefore, we studied the nature of the cells in aggregates and in particular, whether they were neutrophils and/or macrophages. To identify the presence of neutrophils, consecutive sections were analyzed for immunofluorescence staining of neutrophils and for the morphology of focal infiltration point (HE staining). Fig. 3D to 3G show pairs of sections that correspond to the three types of foci described above. All infiltration points corresponding to the first and second types also showed positive neutrophil staining (Fig. 3D and 3E respectively). We found some focal infiltration points corresponding to the third type that were neutrophil-positive (Fig. 3F), although most of them were negative (Fig. 3G).

The peroxidase reaction on the F4/80 macrophage

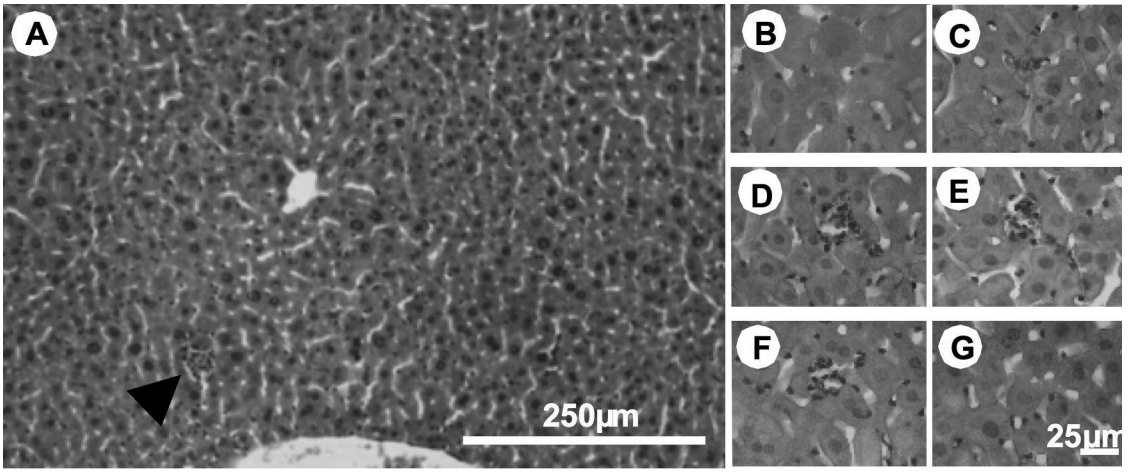

Fig. 1. Leukocyte infiltration foci in liver parenchyma. Liver samples were processed for histological examination. Transverse sections (7 µm thick) of paraffin-embedded livers were stained with hematoxylin-eosin. The arrowhead in panel A points to a leukocyte infiltration focus. **Panels B to G:** serial sections were analyzed to determine the depth of infiltration foci.

antigen was used for macrophage (Kupffer cells) staining. Focal infiltration point morphology was identified by counterstaining with methyl green. Figs. 3H to 3K show representative results. Disperse labeling of Kupffer cells was always observed in liver sections. However, first type focal infiltration points never concentrated macrophages (Fig. 3H). Second type infiltration points could either recruit macrophages (Fig. 3J) or not (Fig. 3I). Third type infiltration points were always macrophage-positive (Fig. 3K). The macrophage antigen is a membrane protein. This explains the clear phagocytosis images (a macrophage membrane surrounding cell debris) that were always observed in this type of infiltration points.

To study whether all types of focal infiltration points increased in parallel after sialoadenectomy we did a new experiment in which we obtained liver samples 2 or 7 days after surgery. We quantified the number of focal infiltration points corresponding to the first, second or third type. As early as 2 days after sialoadenectomy the number of type I infiltration points had increased nearly 5-fold (Fig. 4). The number remained at this high level at day 7. The number of type II infiltration points increased more slowly. Two days after sialoadenectomy it had increased 1.5-fold. The increase in type II infiltration

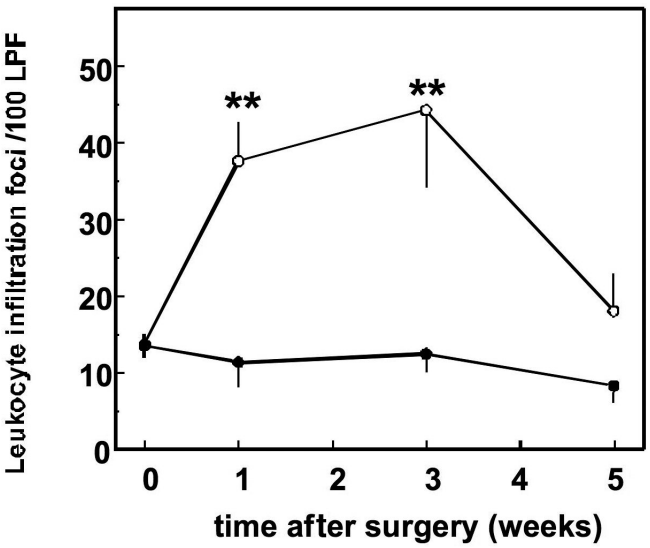

Fig. 2. Sialoadenectomy increases the number of leukocyte infiltration foci in liver parenchyma. Liver samples from control (black dots) and sialoadenectomized (white dots) mice were processed for histological examination. Transverse sections (7 µm thick) of paraffin-embedded livers were stained with hematoxylin-eosin. The number of leukocyte infiltration foci were counted in 20 low-powered fields (LPF: x100 magnification) per slice. Four randomly selected slices per animal were counted. Results are the mean±S.E. of 5 animals per group. The significance of the differences vs the corresponding control value was tested by two-way ANOVA and post-hoc Tukey's test. **: p<0.01.

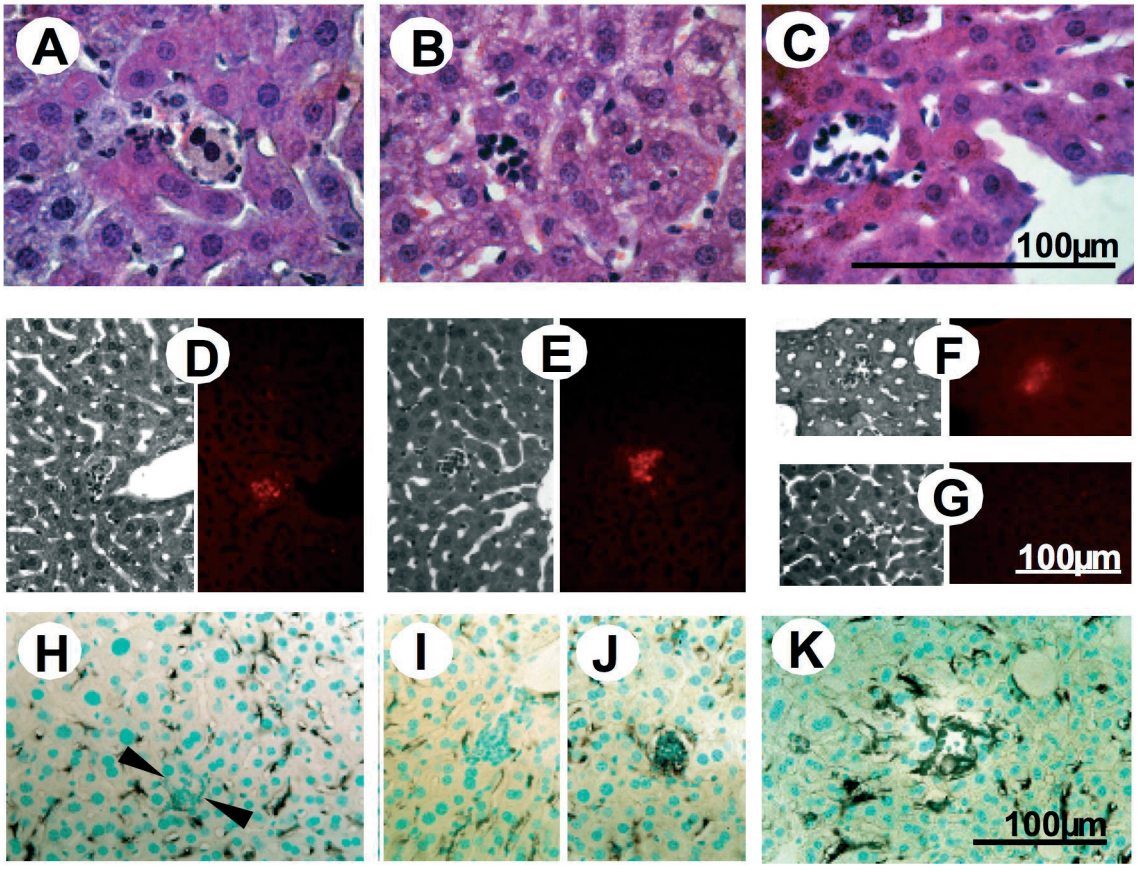

Fig. 3. Characterization of the leukocyte infiltration focus typologies. Liver samples from sialoadenectomized mice were processed for histological examination. Transverse sections (7 µm thick) of paraffin-embedded livers were stained with hematoxylin-eosin (**A-C**). Pairs of consecutive sections (**D-G**) were stained, one with hematoxylin-eosin and the other for immunofluorescence detection of neutrophils. Another set of sections (**H-K**) were processed for the immunohistochemical detection of macrophages (dark label) and counterstained with methyl green.

points was only significant after 7 days. The differences in the number of type III focal infiltration points were not significant. Total number of leukocyte infiltration foci per 100 LPF in sialoadenectomized mice liver was significantly higher both at 2 days (32.6±7.9, $p<0.05$) and 7 days (39.7±5.1, $p<0.001$) than in sham-operated mice (13.6±1.4).

In these animals we measured also liver myeloperoxidase (MPO) (a marker of neutrophil infiltration) and plasma transaminase activities. MPO activity was significantly higher in the liver of sialoadenectomized mice at 7 days after surgery (2.43±0.10 U/g, $p<0.01$) than in sham-operated mice (1.76±0.08 U/g). At 2 days after sialoadenectomy, the increase was not significant (1.86±0.06 U/g). Neither ALT nor AST in plasma were significantly different in sialoadenectomized than in sham-operated mice (data not shown).

Since these results suggest that dying hepatocytes underwent apoptotic but not necrotic death process, we studied the release of cytosolic enzymes (LDH) and the production of tumor necrosis factor-α (TNF-α) and interleukin-1ß (IL-1ß) by isolated livers from sham-operated and sialoadenectomized mice. Both the leakage of intracellular components and the inflammatory response (cytokine production) are characteristics of necrotic cell death. For over an hour, neither control nor sialoadenectomized livers released a measurable amount of TNF-α (Fig. 5). After 90 min, sialoadenectomized livers released an increasing amount of this cytokine. The release of TNF-α stabilized at 0.13 ng/min after 150 min. Control livers did not release more than 0.03 ng/min of TNF-α. Both livers released a low but constant amount of IL-1ß (0.11-0.13 ng/min) from the beginning of the perfusion. After 90 min, sialoadenectomized livers continuously increased the production of this cytokine. At the end of the experiment, the production stood at 0.6 ng/min. The release of LDH, remained constant and low in control livers. LDH in perfusate was not detected until minute 110. Thereafter, it increased rapidly in livers from sialoadenectomized mice and more slowly in livers from control mice.

All these curves fit a logistic sigmoid: $Y = Min + (Max-Min)/(1+e^{(-k(t-t_{50}))})$, where *Max* and *Min* are

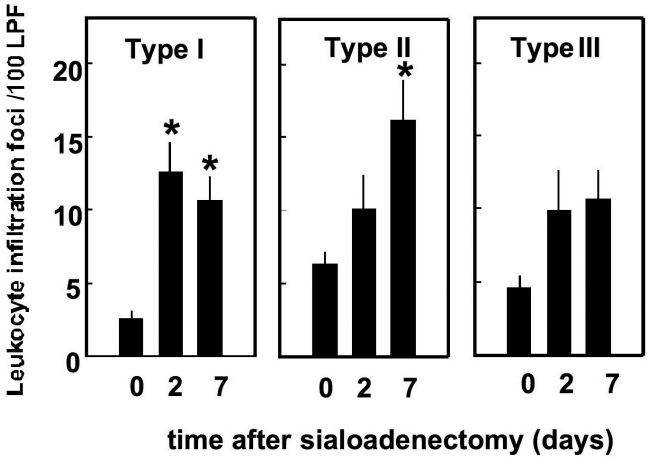

Fig. 4. Early effect of sialoadenectomy on the number of leukocyte infiltration focus types in the liver. Liver samples were processed for histological examination. Transverse sections (7 Ìm thick) of paraffin-embedded livers were stained with hematoxylin-eosin. The number of leukocyte infiltration foci corresponding to type I, II and III were counted in 20 low-powered fields (LPF: x100 magnification) per slice. Four randomly selected slices per animal were counted. Results are the mean±S.E. of 10 animals per group. The significance of the differences vs the control value was tested by two-way ANOVA and post-hoc Tukey's test. *: $p<0.05$.

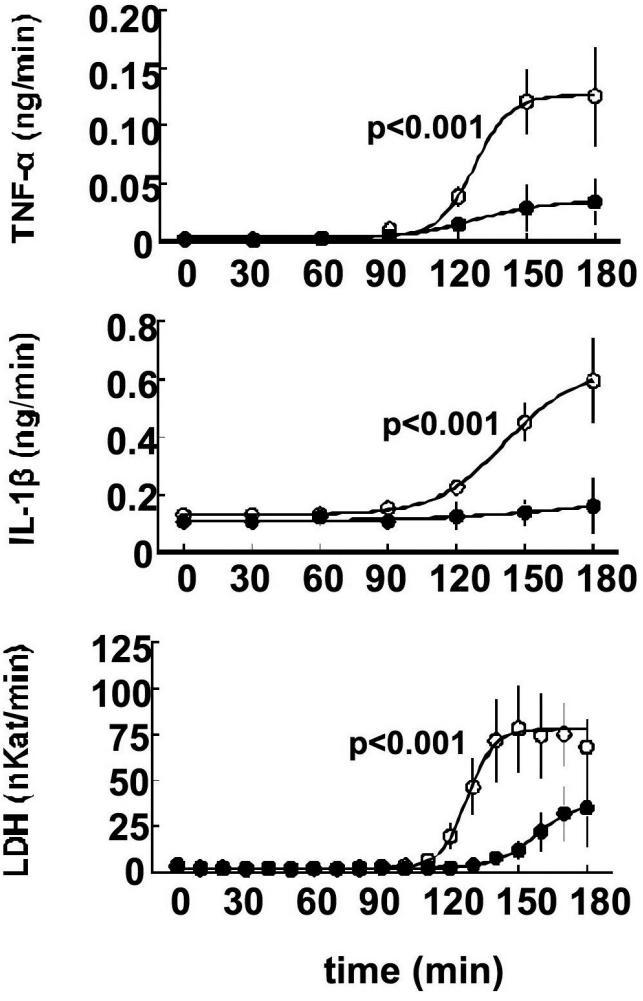

Fig. 5. Effect of sialoadenectomy on the production of cytokines and the release of LDH by isolated livers. Isolated livers from sham-operated (black dots) or sialoadenectomized (white dots) mice were perfused in non-recirculation conditions. Samples of the perfusate were obtained to measure TNF-α, IL-1ß, and LDH. The results are the mean±S.E. of 6 animals per group. The significance of the differences was tested by one-way ANOVA.

maximal and minimal values respectively, *k* is a rate constant and t_{50} is the time to reach the half-maximal value. All these parameters could be precisely estimated in livers from sialoadenectomized mice but not from control animals. It is interesting that t_{50} for TNF-α production was 127±3 min, which is identical to the t_{50} value for LDH release: 127±1 min. The t_{50} for IL-1β production was higher: 141±1 min. Accordingly, there was a high correlation (r=0.885, p<0.001) between the rate of LDH release during the perfusion and the rate of TNF-α production (Fig. 6).

To study whether the alterations described above in liver parenchyma of sialoadenectomized mice could be attributed to a transient lack of ErbB1 activation because of the transient disappearance of EGF in the plasma of sialoadenectomized mice (Buira et al., 2004), we looked at the effect of 3 day administration of the ErbB1 inhibitor genistein. In a preliminary experiment we injected either 1 or 5 mg genistein/mouse and studied 12 h later whether an acute injection (i.p.) of EGF could induce tyrosine phosphorylation of the 170 kD band corresponding to ErbB1. As shown in Fig. 7A, EGF induced the appearance of a phospho-Tyr containig protein of 170 kD. No other phosphorylated bands were observed in the high molecular weight range (not shown). Genistein (5 mg/mouse) prevented the appearance of this phosphorylated band. At the lower dose (1 mg/mouse) this band appeared but at lower intensisty. In the next experiment, genistein (5 mg/mouse) was injected twice/day during 3 consecutive days. The number of leukocyte infiltration foci in liver sections of genistein-treated mice were nearly 3-fold

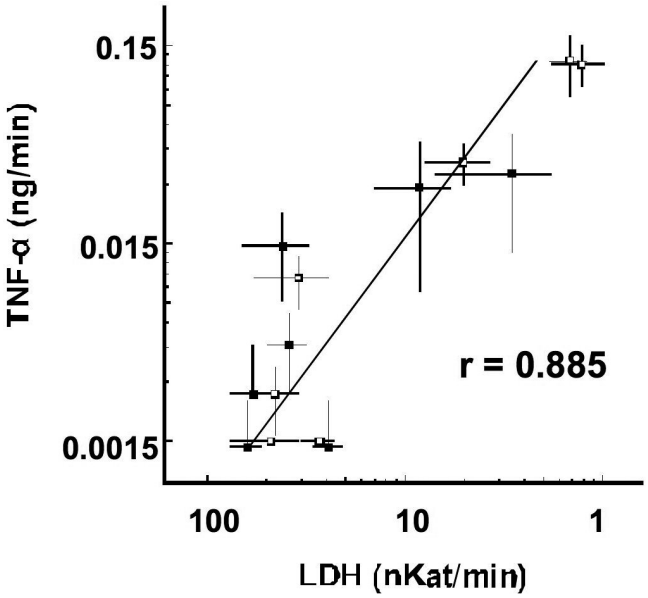

Fig. 6. Correlation between LDH release and TNF-α production. Mean values of LDH release shown in Fig 5 were plotted against those of TNF-α production.

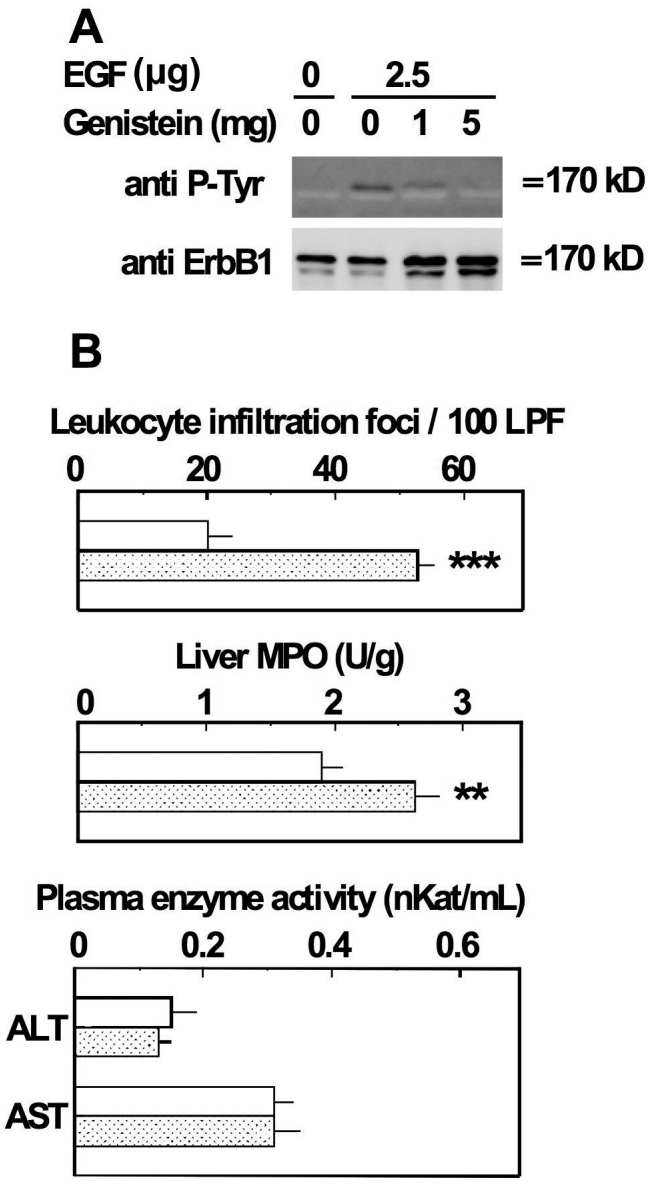

Fig. 7. Genistein increases the number of leukocyte infiltration foci in liver parenchyma. In the preliminary experiment (**panel A**) mice received a single s.c. injection of genistein (1 or 5 mg/mouse) or the vehicle (ricine oil, 0.05 mL). Twelve h later The animals were injected (i.p.) with EGF (2.5 µg/mouse) or the vehicle (PBS, 0.1 mL). Clarified liver homogenates (10 µg protein/lane) were subjected to polyacrylamide-gel electrophoresis and electro-blotting to nylon membranes. Tyrosine phosphorylated proteins were visualized with an anti P-Tyr antibody and the EGF receptor with an anti ErbB1 antibody. **Panel B:** Mice were injected (3 days) with 5 mg genistein/12 h/mouse (dotted bars) or the vehicle (ricine oil, white bars). Twelve h after the last injection mice were sacrificed. Liver samples from were processed for histological examination. Transverse sections (7 ìm thick) of paraffin-embedded livers were stained with hematoxylin-eosin and the number of leukocyte infiltration foci were counted in 20 low-powered fields (LPF: x100 magnification) per slice. Four randomly selected slices per animal were counted. Another liver sample was processed to determine myeloperoxidase (MPO) activity. Transaminase (ALT and AST) activities were determined in plasma samples. Results are the mean±S.E. of 5 animals per group. The significance of the differences vs the control value was determined by Student's t-test. **: p<0.01; ***: p<0.001.

higher than in oil-injected mice (Fig. 7B). In accordance, MPO activity was also increased in liver samples of genistein-treated mice. However, genistein did not affect plasma transaminase activities (ALT and AST).

We showed previously (Buira et al., 2004) that the administration of bacterial lipopolysaccharide (LPS) (combined with galactosamine: Gal-N) to sialoadenectomized mice (3 weeks after surgery) resulted in a greater increase in plasma transaminase activities than in control mice. To determine the relevance of this

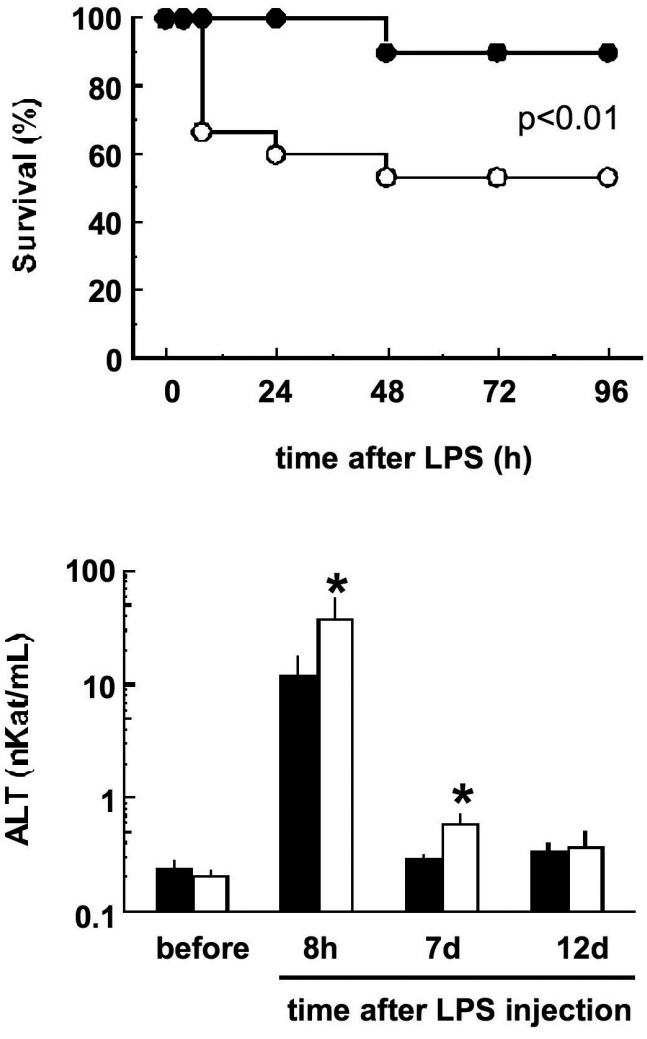

Fig. 8. Effect of sialoadenectomy on the response to an endotoxin burst. Ten sham-operated (black dots and bars) or sialoadenectomized (white dots and bars) mice received an ip injection of lipopolysaccharide (100 µg/Kg) and D-galactosamine (750 mg/Kg). Survival was monitored during the next 96 h (upper panel). Survival curves were analyzed by Kaplan-Meier and the differences tested by the log Rank test. Lower panel: blood samples were obtained from the tail vein at the indicated times (8h and 7 and 12 days), in order to determine ALT activity. The significance of the differences vs the control value was tested by two-way ANOVA and post-hoc Tukey's test. *: p<0.05.

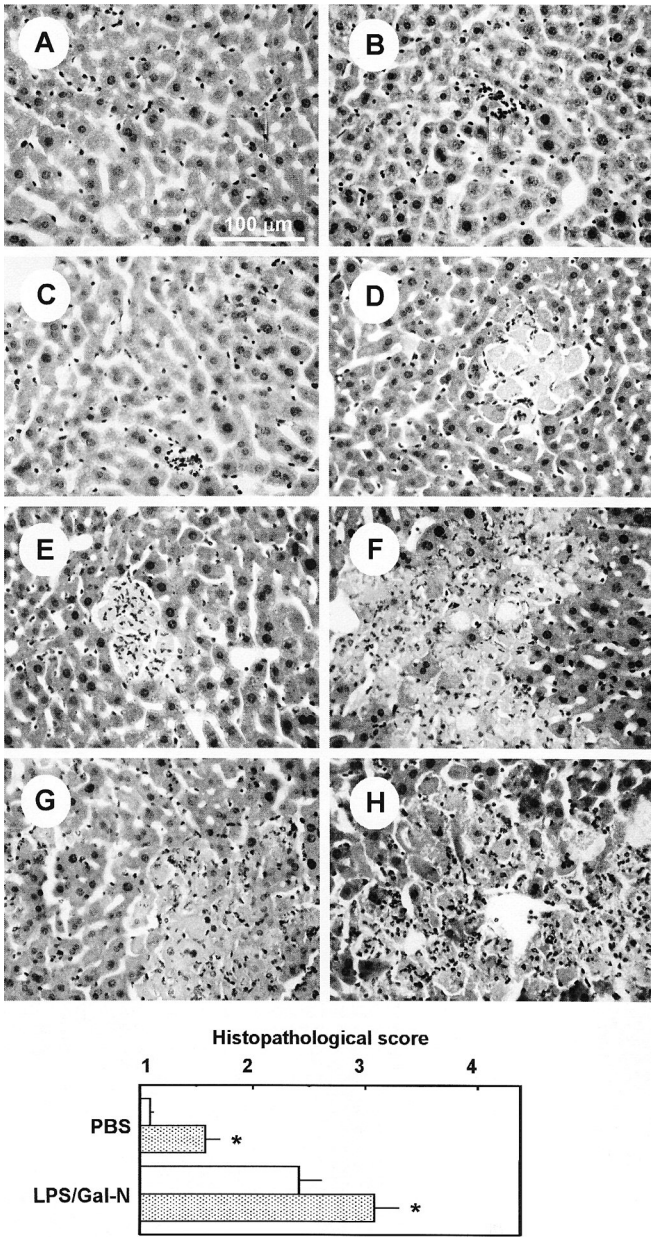

Fig. 9. Effect of sialoadenectomy on liver injury induced by an endotoxin shock. Sham-operated (panels **A, C, E, G** and white bars) or sialoadenectomized (panels **B, D, F, H** and dotted bars) mice received an ip injection of lipopolysaccharide (10 µg/Kg) and D-galactosamine (750 mg/Kg) and were sacrificed 24 h later. Control animals received PBS. Liver samples were processed for histological examination. Transverse sections (7 µm thick) of paraffin-embedded livers were stained with hematoxylin-eosin. Histopathological score (1 to 4) was determined in 20 high-powered fields (HPF: x 400 magnification) per slice. Four randomly selected slices per animal were analyzed. Results are expressed as the mean±S.E. of 5 animals per group. The significance of the differences between sham-operated and sialoadenectomized mice were determined by the Mann-Witney test. *, p<0.05.

Table 1. Properties of the different stages in the hepatocyte death process.

	Stage I	Stage II	Stage III
cell density	low	high	low
dying hepatocyte	visible	not visible	not visible
neutrophils	+	+	- / +
macrophages	-	- / +	+

This table summarizes the properties of the different types of leukocyte infiltration foci observed in the liver of both control and sialoadenectomized mice.

consequence of sialoadenectomy, we studied the effect of a higher dose of lipopolysaccharide (100 µg/Kg) combined with galactosamine (750 mg/Kg) on mouse survival. Most control mice (90%) survived at this dose of LPS (Fig. 8, upper panel). However, only 50% of sialoadenectomized animals survived. Surviving animals in both groups were allowed to recover for 12 days and ALT activity in plasma was assayed at several time points. In preliminary experiments, we found that the highest ALT activity in plasma was obtained 8 h after LPS administration (data not shown). Therefore, we measured plasma ALT activity at 8 h and then at 7 and 12 days. At the peak time, this activity was 3-fold higher in surviving sialoadenectomized than in control mice (Fig. 8, lower panel: note the logarithmic ordinate axis). Obviously, this is consistent with the higher mortality that occurred at this time. Seven days after the LPS burst, the plasma ALT in control mice was completely recovered, whilst in sialoadenectomized mice it was still significantly higher. After 12 days, normality was also recovered in sialoadenectomized mice.

To obtain direct evidence that the hepatotoxic effect of LPS/Gal-N was exacerbated in sialoadenectomized mice, we looked at liver sections of both sialoadenectomized and sham-operated mice. This study was performed in animals receiving a lower dose of LPS (10 µg/Kg) to avoid the effect of the higher mortality shown above. At the dose of 10 µg/Kg no animal (either sham-operated or sialoadenectomized) died. The analysis of HE stained sections of livers showed a higher degree of injury in samples from sialoadenectomized (Fig. 9B, D, F, H) than in those from sham-operated mice (Fig. 9A, C, E, G) injected with LPS, but also a very high degree of variability between different animals. Thus, to get significant conclusions we performed a quantitative analysis by scoring (1 to 4) the images obtained from liver sections. Fig. 9A was scored 1 and corresponded to non-affected area. Fig. 9B and C were scored 2 and corresponded to single dying hepatocytes with focal or diffuse leukocyte infiltration. Figs. 9D and E were scored 3 and corresponded to a small group of damaged hepatocytes. Fig. 9F-H were scored 4, they corresponded to large necrotic areas. In sialoadenectomized mice injected with PBS, the mean score was higher than in corresponding sham-operated animals (Fig. 9, lower panel). This is in keeping with the quantification of leukocyte infiltration foci shown in Fig. 2. Any HPF image was scored 3 or 4 in these animals. In sham-operated mice injected with LPS/Gal-N most sections scored 2-3, while in sialoadenectomized mice a significant proportion of sections scored 4. The statistical analysis revealed that the mean score was significantly higher in sialoadenectomized than in sham-operated mice.

Discussion

In a previous study we found that the number of TUNEL-positive hepatocytes increased transiently after sialoadenectomy (Buira et al., 2004). Here we show that neutrophils and macrophages (Kupffer cells) participated in a sequential manner in the removal of dying hepatocytes. Based on the morphology of the focal infiltration points and on the presence of neutrophils and macrophages, we identified three stages in the removal process. Table I shows the characteristics of each stage.

Our results suggest that early after commitment to apoptotic death, and at the time of chromatin condensation, neutrophils are recruited on dying hepatocytes. It was recognized that hepatocyte apoptosis in models of endotoxin-induced and Fas-antibody-induced liver injury generates signals for the sequestration and transendothelial migration of neutrophils (Lawson et al., 1998). These signals may involve CXC chemokines (Dorman et al., 2005). It is likely that neutrophils contribute to the progression of the apoptosis process by generating oxidative stress (Jaeschke, 2006). We suggest that this early stage evolves to stage II, which is characterized by a dense label of neutrophils and by the absence of a whole hepatocyte. It is conceivable that, in addition to neutrophils, residual apoptotic bodies are present in this stage. Since macrophages were not always observed at this stage, we suggest that these cells accumulate in the periphery of the infiltration focus later than neutrophils. The phagocytic function of Kupffer cells explains why void spaces are observed in stage III. This process may end with the complete clearance of residual bodies. Neighboring hepatocytes will ultimately occupy this space. This is in keeping with our previous observations that DNA synthesis increases in the liver of sialoadenectomized mice, and that the mean hepatocyte volume is 60% higher in sialoadenectomized than in control mice (Buira et al., 2004). Taken together, these findings suggest that a cellular hypertrophy process occurs to occupy the place of dead hepatocytes.

The sequential increase in the number of type I, II and III infiltration foci early after sialoadenectomy argues in favor of the proposed process for the clearance of dying hepatocytes, and points to neutrophil infiltration playing a pivotal role. The role of neutrophils in acute inflammatory liver injury is well known (Jaeschke and Hasegawa, 2006). These cells are commonly associated with the extension of liver damage and necrotic death, as

in ischemia-reperfusion injury (Jaeschke et al., 1990), endotoxin shock (Jaeschke et al., 1991) alcoholic hepatitis (Bautista, 1997), obstructive cholestasis (Gujral et al., 2003) and remote organ trauma (Vega et al., 1999).

Sialoadenectomy caused mild liver damage. There was no evidence of necrotic death. We did not observe an extension of the damage into neighboring cells and there was no increase in plasma transaminase activities. The experiments with perfused livers further support the conclusion that the liver of sialoadenectomized mice does not contain necrotic foci: for over an hour, neither control nor sialoadenectomized mouse livers released any measurable amount of TNF-α or LDH. However, in perfused liver from sialoadenectomized mice hepatocytes are more susceptible to the initiation of a death process. This is indicated by the delayed increase in both parameters and in the production of IL-1ß.

Hepatocytes damaged by long-term perfusion conditions cannot recruit neutrophils in an isolated liver. In addition, conditions that promote cell death in a whole animal may be different to those that damage cells in the perfusion system. Thus, it is conceivable that hepatocytes initiating a death process may follow a different pathway in the perfusion system than in the whole animal. In perfused liver, dying cells release intracellular content (LDH) into the medium, indicating that the necrotic process was taking place. This may immediately activate Kupffer cells, which respond with the production of TNF-α. Note that we found a good correlation between the rate of LDH release and the rate of TNF-α production throughout the perfusion. The production of IL-1ß follows delayed kinetics. This is in keeping with several experiments in whole animals that were challenged with bacterial endotoxin (Haddad et al. 2001; Matsuzaki et al. 2001). This may explain why the correlation between the production of IL-1ß and the release of LDH is worse than that between TNF-α and LDH (data not shown).

We suggest that the transient increase of hepatocyte death (number of infiltration foci) in the liver of sialoadenectomized mice may be the consequence of the lack of EGF production from submandibular salivary glands. First, because it is coincident with the drop in plasma EGF concentration (Buira et al., 2004). Second, because genistein, an inhibitor of EGF receptor (ErbB1) tyrosine kinase activity (Akiyama et al., 1987), produced a similar effect to those of sialoadenectomy; an increase of both leukocyte infiltration foci and MPO activity in the liver. The results presented here, and previous reports (Grau et al., 1994; Buira et al., 2004) point to a submandibular salivary glands-liver axis whose function may be the maintenance of liver cellularity.

EGF is a potent antiapoptotic (surviving) factor for hepatocytes (Fabregat et al., 1996). Hepatocyte survival is controlled by the balance between antiapoptotic (including EGF) and apoptotic factors (Roberts et al., 2000; Ethier et al., 2003; Musallam et al., 2004). Therefore, it is conceivable that the transient lack of EGF leaves hepatocytes more susceptible to the initiation of a death process. Although it involves recruitment of neutrophils, the process appears to be kept under control and the subsequent accumulation of macrophages (Kupffer cells) ends in the complete removal of residual bodies.

The damage to the liver caused by sialoadenectomy is very mild. However, the consequences are significant: the hepatotoxic effect of an endotoxin burst is axacerbated. Furthermore, survival after an otherwise sublethal endotoxin burst decreased in sialoadenectomized mice. Surviving sialoadenectomized mice recovered more slowly than controls, as indicated by the high plasma ALT activity a week after the burst. Several stressful conditions induce mild liver damage (Salas et al., 1980; Adachi et al., 1993; Sánchez et al., 2002). It was described that stressed animals are more susceptible to endotoxin shock (Quan et al., 2001; Sánchez et al., 2007) and to ß-galactosylceramide- or CCl_4-induced injury (Chida et al., 2004; Panuganti et al., 2006). We conclude that although the alterations in liver structure induced by sialoadenectomy are mild, they have delayed consequences on the ability of the liver to deal with aggressive insults.

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