# Administration of *Escherichia coli* endotoxin to rat increases liver mass and hepatocyte volume *in vivo*

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We have established, *in vivo*, an increase in liver mass and hepatocyte volume after a single intraperitoneal administration, to fasted rats, of *Escherichia coli* lipopolysaccharide (0127:B8) at 3 mg/kg. The phenomenon was time- and dose-dependent and could be prevented by treatment with polyclonal antiserum against tumour necrosis factor-$\alpha$ (TNF-$\alpha$) before the endotoxin injection. Endotoxin caused an increase of 26% in the hepatic mass compared with fasted controls at 24 h. An increase of 27% in the hepatic water content underlay the altered hepatic mass which could not be accounted for by a change in the volume of hepatic blood and/or interstitial fluid (measured *in vivo*), suggesting an expansion in the hepatocellular volume. This is supported by an increase of 25% in the $K^+$ content of the endotoxic livers. Morphometric study confirmed a 15% increase in hepatocyte volume after endotoxin administration. The data are discussed in the light of possible metabolic effects of increased hepatocyte volume.

## INTRODUCTION

Recent studies have implicated altered liver cell volume and/or volume regulatory responses in the regulation of metabolism in the liver. Increased hepatocyte volume has been associated with stimulation of hepatic glycogen synthesis, amino acid uptake, protein synthesis and ureogenesis from amino acids and inhibition of hepatic glycogenolysis, glycolysis and proteolysis [1]. These studies have, in general, been carried out with *in vitro* preparations. There are few published studies on altered hepatocyte volume *in vivo*.

A number of studies have described significant increases in liver mass *in vivo* in experiments with animal models of sepsis [2], endotoxaemia [3], trauma [4], chronic inflammation [5], or after administration of recombinant tumour necrosis factor-$\alpha$ (TNF-$\alpha$) [6], but the focus of those studies was largely on changes in protein synthesis or lipid metabolism. In these studies, the net increase of hepatic protein content or lipid content reported could not account for the increase in the liver mass; which cellular compartment(s) are responsible for the enlargement of liver has not been elucidated. In the present study, we investigated the early increase in liver mass that occurs *in vivo* upon administration of *Escherichia coli* lipopolysaccharide (LPS) to rats and found a substantial increase in hepatocyte volume.

## MATERIALS AND METHODS

### Materials

*E. coli* LPS (endotoxin; serotype 0127:B8, phenol extraction) was obtained from Sigma Chemical Inc. (St. Louis, MO, U.S.A.). The serum IgG fraction of goat polyclonal antiserum against recombinant murine TNF-$\alpha$ (anti-TNF-$\alpha$, 22.8 mg/ml) and control goat IgG (IgG, 20.5 mg/ml) were gifts from Dr. Gregory Bagby (Department of Physiology, Louisiana State University Medical Center). This antibody does not cross-react with LPS, interleukin (IL)-1 or interferon-$\gamma$ [7]. NaCl (0.9%) injection is a pyrogen-free product. [$^{14}$C-*carboxyl*]Inulin ($M_r$ 5000–5500) was obtained from Dupont New England Nuclear Corp. (Mississauga, Ontario, Canada). All enzymes and coenzymes were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Boehringer Mannheim GmbH (Dorval, Quebec, Canada). All other chemicals used were of analytical grade.

### Animal model

Adult male Sprague–Dawley rats (Charles River, Montreal, Quebec, Canada) weighing 250–350 g were used throughout. Before the experiment, rats were conditioned at $22 \pm 2$ °C with free access to tap water and rat chow (Ralston Purina of Canada, Don Mills, Ontario, Canada) for at least 7 days under a reversed 12 h/12 h-light/dark cycle (dark period was from 8:00–20:00 h).

In the course of preliminary studies, we found that fed rats were not an appropriate control for the endotoxin-treated rats which, when fed *ad libitum*, manifested a profound decrease in spontaneous food intake and had distended stomachs with marked food stagnation. This is consistent with the published reports that endotoxin administration constricts the pyloric sphincter and thus inhibits gastric emptying [8,9]. In order to maintain the same post-absorptive state we therefore fasted both the endotoxin-and saline-treated group. A group of normal-fed rats was also included.

### Experimental protocol

On the experiment day, the rats in the normal-fed group were allowed rat chow *ad libitum* until the surgery; the rats in all other groups were deprived of food 1–2 h (6:00–7:00 h) before the onset of the dark period to ensure that their stomachs were empty when injected with endotoxin or saline. They remained fasted during the entire experiment. In each experiment the rats were matched for body weight such that the initial weights were within 5%.

Abbreviations used: TNF-$\alpha$, tumour necrosis factor-$\alpha$; LPS, lipopolysaccharide; i.p., intraperitoneal; TCA, trichloroacetic acid; PMNs, polymorphonuclear leucocytes; IL, interleukin; PEPCK, phosphoenolpyruvate carboxykinase.

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The treatments for the fasted rats in the different groups were as described below.

(1) Endotoxin- and saline-treated group: 4 h after the deprivation of food (10:00–11:00 h), initial body weights were recorded. The rats in the endotoxic group were injected with *E. coli* LPS in pyrogen-free saline [1 mg/ml, 3 mg/kg, intraperitoneal (i.p.) injection or at various doses as required in dose–response experiments]. The rats in the saline-treated group were injected with pyrogen-free saline (3 ml/kg, i.p.) as controls.

(2) Anti-TNF-$\alpha$ + LPS group and control IgG + LPS group: the rats in both groups were injected with the endotoxin as described above for the endotoxic group. Two hours prior to the injection of endotoxin (8:00–9:00 h), they were treated with either goat anti-TNF-$\alpha$ IgG (20 mg/300 g rat, i.p.) or control goat IgG (20 mg/300 g rat, i.p.).

The liver was removed by complete hepatectomy at 24 h after the injection or at various times in the time-course experiment.

### Determination of liver mass and hepatic components

#### 1. Hepatectomy

Complete hepatectomy was used to remove the whole liver without loss of blood or tissues. The surgical technique was based on the procedures described by Waynforth [10] with modification. The rat was anaesthetized with sodium pentobarbital, 60 mg/kg i.p., and placed on a heating pad. After a mid-line laparotomy, the gut was deflected to the rat's left to expose and locate the liver lobes and splanchnic vessels. Suspensory ligaments were then cut down as near as possible to the blood vessels using blunt-ended curved scissors. The portal vein, hepatic artery and inferior vena cava were exposed by blunt dissection. Silk surgical threads (4/0) were loosely placed around these vessels. They were simultaneously ligated and the liver was removed by severing the blood vessels at the sites of these ligatures. Finally, the non-hepatic tissues attached were excised. The time between the mid-line incision and severance of all blood vessels was less than 10 min.

#### 2. Total liver mass and liver wet/dry weight ratio

The liver removed by hepatectomy was weighed and the hepatosomatic index (liver weight as a percentage of the final body weight), were calculated. Hepatic water content and wet/dry weight ratio were measured by drying a portion of the liver, at 55–60 °C, to a constant weight.

#### 3. Analysis of hepatic dry mass components

A portion of the liver obtained from the hepatectomy was freeze-clamped in liquid nitrogen, then ground to a fine powder and extracted by different procedures to measure the hepatic content of protein, lipids, DNA and potassium. In a series of separate experiments, the liver tissues was rapidly freeze-clamped for the assay of hepatic glycogen and adenine nucleotides.

### Determination of hepatic extracellular space *in vivo*

Water comprises about 70 % of the total liver weight. The total hepatic fluid compartment can be divided into hepatic cellular and extracellular space. We determined the volume of hepatic cellular space by subtracting the extracellular space from the total hepatic water content. $^{14}$C-labelled inulin was chosen as an extracellular space marker, assuming that it distributes freely in the extracellular space and it is not taken up by liver cells. This latter point was checked by preliminary experiments which showed that 93.0 $\pm$ 1.7 % ($n = 3$) of the hepatic [$^{14}$C]inulin that had been infused *in vivo* was released by flushing the liver with 200 ml of saline.

Rats were anaesthetized and treated as for the hepatectomy. A priming dose of 8 $\mu$Ci of [$^{14}$C]inulin in 0.8 ml of 0.9 % NaCl was injected through the left saphenous vein and followed immediately by continuous infusion of 5 $\mu$Ci of [$^{14}$C]inulin in 1.5 ml of 0.9 % NaCl at a rate of 0.037 ml/min. Preliminary experiments (results not shown) showed that the plasma [$^{14}$C]inulin level was maintained at a plateau between 20 and 40 min. After 25 min, 0.15 ml of heparin (1000 USP units/ml) was injected via the left saphenous vein and at 30 min, 0.2–0.3 ml of blood was slowly withdrawn from the hepatic portal vein. A portion of this blood was used for haematocrit determination and 20 $\mu$l of blood was used for haemoglobin determination. Plasma, obtained from the remaining blood, was used for $^{14}$C measurement. The whole liver was removed by complete hepatectomy (immediately after the blood sample was taken from the portal vein) and was homogenized with 20 ml of deionized water. The homogenate was frozen overnight, and, after thawing, was centrifuged at 120000 ***g*** for 60 min at 4 °C. An aliquot of the supernatant was used for haemoglobin determination. Another aliquot was deproteinized with an equal volume of 0.5 M $HClO_4$ and $^{14}$C was determined by scintillation counting of the deproteinized extract.

The hepatic extracellular space is the sum of interstitial fluid plus plasma volume. The total volume of hepatic blood was calculated from the haemoglobin content of the liver and of the portal venous blood. The hepatic plasma volume and the volume occupied by blood cells was obtained from the haematocrit and the total hepatic blood content. The total [$^{14}$C]inulin content of the liver is a measure of the hepatic extracellular space. Subtraction of the plasma space from the extracellular space gives the interstitial space.

### Morphometric study of hepatocytes

Morphometry of hepatocytes was used as an additional method to determine changes in the volume of the hepatic cellular space. Rats were anaesthetized as for the hepatectomy and 0.5–1.0 g of liver tissue excised from the left lateral lobe was immediately diced into 1 mm $\times$ 1 mm blocks. The tissue blocks were pooled and fixed in buffered Karnovsky's fixative (pH 7.4) at room temperature [11]. Four to five fixed blocks were chosen at random and first dehydrated and defatted by a series of treatments with sodium cacodylate, osmium tetroxide, uranyl acetate, ethanol and acetone [12]. Each block was then embedded, dried, polymerized and sectioned serially at 0.5–1.0 $\mu$m.

Four to five technically perfect sections chosen from each block were finally stained for 1 min at 60 °C with 1 % Toluidine Blue.

The morphometry of hepatocytes was performed by a 'blind' investigator, unaware of the treatment of the animals, using an image analysis system (BIOQUANT System IV, R & M Biometrics, Inc.). At the $\times$10 magnification level, an area large enough to include two hepatic lobules (each with a central vein at the centre) was chosen as the representative structure of the liver. At the $\times$40 magnification level, several adjacent microscope fields within the portal area were chosen provided they met the requirement of sufficient resolution for unambiguous recognition of the cell boundary. The micrograph of each individual microscope field was displayed on the monitor with reversed enhancement, which minimized the background interference and optimized the contrast of hepatocyte outline. The outline, i.e. the perimeter, of each individual mononuclear hepatocyte (150–250 in total for each liver) with a clear and intact nucleus was then traced on the screen by a visible cursor. The images were

digitized and integrated by the BIOQUANT system to give the perimeter and transectional area of each individual hepatocyte. The number of all binucleate and mononucleate hepatocytes within the microscope fields chosen were also counted to give the percentage of binucleate hepatocytes of each liver. The shape factor, which helps to identify morphometric variations in different cell populations, was also calculated by the BIOQUANT system. It is the ratio of the shortest diameter over the longest diameter of each hepatocyte on the transectional profile (where 0 = a straight line; 1 = a perfect circle).

Assuming each hepatocyte to be approximately spherical, the volume ($V$) of an individual hepatocyte can be calculated from its radius ($r$) by the equation $V = 4\pi r^3/3$. Since the transectional area ($A$) of each hepatocyte cell body is given by the BIOQUANT system, and $A = \pi r^2$, the average $r$ can be calculated. The hepatocyte volume ($V$) in the morphometric data is then given by the equation $V = 4rA/3$ after substitution of $\pi r^2$ with $A$ in the equation $V = 4\pi r^3/3$.

### Other analytical methods

Glycogen was extracted from frozen liver powder and hydrolysed to glucose, which was determined by the glucose oxidase method (Sigma kit 510-A). Amino acid analyses in blood and frozen liver powder were performed by ion-exchange chromatography [13]. Hepatic protein content was determined by the biuret method with BSA as standard. Total hepatic lipids were measured by the gravimetric method [14] with modifications. Assays for hepatic potassium content were carried out by atomic absorption spectrophotometry [15]. Hepatic DNA was extracted by trichloroacetic acid (TCA) as described by Enesco and Leblond [16] except that the successive washings of the TCA-extracted pellet by distilled water and absolute ethanol were omitted. DNA was then determined by Burton's method [17]. Single-stranded calf thymus DNA was used as standard. Hepatic adenine nucleotides were determined in rapidly freeze-clamped liver, as described by Hems and Brosnan [18]. The activity of serum alanine aminotransferase was determined according to Bergmeyer and Bernt [19]. Haemoglobin content was determined by the cyanomethaemoglobin method [20].

### Statistical analysis of data

Results are expressed as means $\pm$ S.D. with the number of rats in parentheses. A significant difference between two individual means was determined by an unpaired $t$-test as appropriate. Significant differences among three or more individual means were determined by one-way analysis of variance followed by the Bonferroni $t$-test for multiple comparisons. A $P$ value of less than 0.05 was taken to indicate statistical significance.

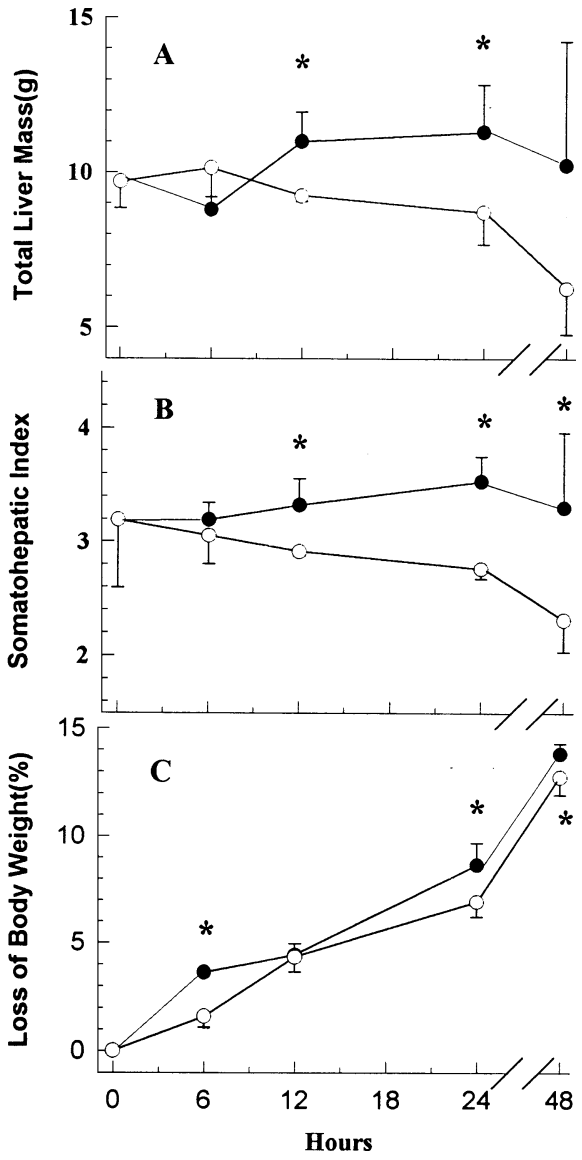


**Figure 1 Temporal changes in liver mass (A), hepatosomatic index (B) and body weight (C) after endotoxin administration**

The mean initial body weights were 321 $\pm$ 36 g and 332 $\pm$ 28 g for endotoxin-treated (LPS 3 mg/kg, i.p.) (●) and saline-treated control rats (sterile saline 3 ml/kg, i.p.) (○), respectively. The final body weights at death were 300 $\pm$ 31 g and 308 $\pm$ 32 g for the two groups respectively. All values are means $\pm$ S.D. with 3–5 rats in each group at various times, except for 7 rats in each group at 24 h. * $P < 0.05$ versus the time-matched controls.

## RESULTS

### Temporal and dose-dependent increase of liver mass after endotoxin administration

A bolus i.p. injection of *E. coli* LPS resulted in a marked increase in total liver mass (Figure 1A) and hepatosomatic index (Figure 1B). During a 48-h time course, the endotoxin-treated rats lost slightly more body weight (13.84 $\pm$ 0.47 %) than the saline-treated control rats (12.76 $\pm$ 0.81 %) (Figure 1C). The hepatosomatic index in the endotoxic group at 12 h (3.33 $\pm$ 0.22), 24 h (3.50 $\pm$ 0.22) and 48 h (3.41 $\pm$ 0.68), was 17 %, 34 % and 48 % higher than their time-matched saline-treated controls (2.91 $\pm$ 0.04, 2.61 $\pm$ 0.25 and 2.31 $\pm$ 0.28) respectively.

The endotoxin-induced increase in the hepatic mass and hepatosomatic index, measured 24 h after treatment, was generally dose-dependent between 0.03 and 3.0 mg/kg i.p. (Figures 2A and 2B). A dose of 0.3 mg/kg i.p. was the smallest dose required to introduce an unambiguous change in the liver mass. The dose used for this study (3.0 mg/kg, i.p.), which is equivalent to the subcutaneous $LD_{10}$ dose for *E. coli* LPS of the same serotype [3], caused a large increase in the liver mass and was well tolerated by the animals.

### Changes in hepatic water content and dry mass components

Table 1 shows changes in water content and various dry mass components of the liver. After endotoxin administration (24 h),

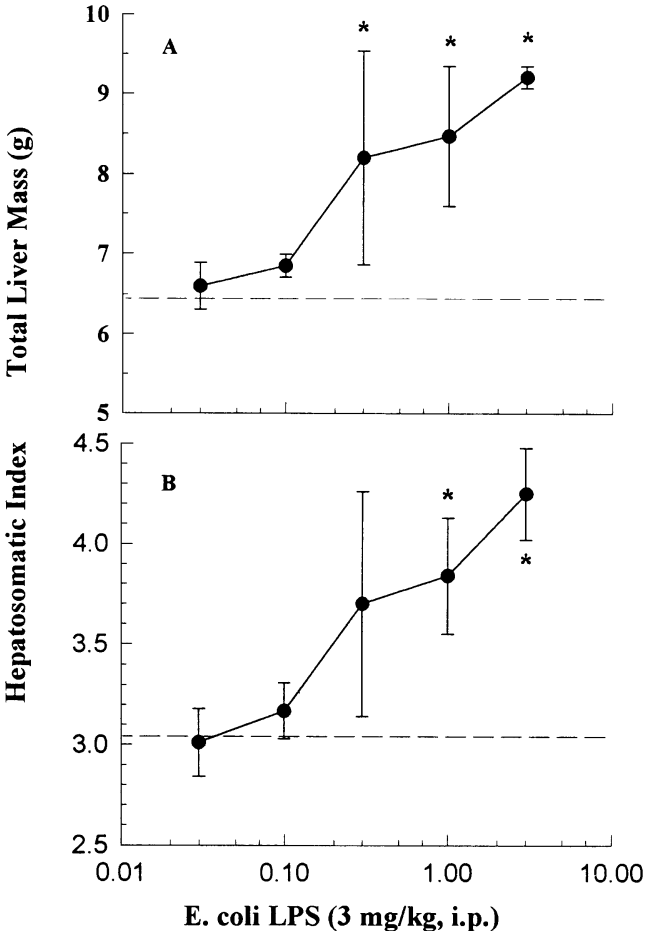


**Figure 2 Dose–response curve of the increase in the liver mass (A) and hepatosomatic index (B) after endotoxin administration**

These studies were performed 24 h after endotoxin administration at doses of 0.03–3.0 mg/kg, i.p. All values are means ± S.D. with 3 rats for each dose, except for 0.3 mg/kg and 1.0 mg/kg where 5 rats were used. * $P < 0.05$, versus corresponding values (- - -) of saline-treated control group (6.49 ± 0.79 g for liver mass and 3.07 ± 0.38 for hepatosomatic index, $n = 4$ for all groups).

**Table 2 Hepatic cellular and extracellular space**

The volume of extrahepatocellular water was calculated by adding the volume of the hepatic interstitial space to the volume occupied by hepatic blood content. The hepatocellular water was calculated by subtracting the volume of the extrahepatocellular water from the total hepatic water content. Data were obtained 24 h after the injection of endotoxin (3 mg/kg, i.p.) or saline (3 ml/kg, i.p.). Values are means ± S.D. with the number of rats in parentheses. * $P < 0.05$ versus saline-treated control group.

| Parameter (per liver) | Saline-treated control ($n = 5$) | Endotoxin-treated ($n = 7$) |
|---|---|---|
| Total liver mass (g) | 10.08 ± 0.67 | 12.84 ± 0.51* |
| Water content (g) | 7.11 ± 0.49 | 9.36 ± 0.81* |
| Inulin space (ml) | 1.57 ± 0.43 | 2.13 ± 0.42 |
| Blood content (ml) | 1.79 ± 0.29 | 1.72 ± 0.22 |
| Plasma space (ml) | 0.81 ± 0.17 | 0.87 ± 0.13 |
| Interstitial space (ml) | 0.77 ± 0.28 | 1.26 ± 0.49 |
| Extrahepatocellular water (ml) | 2.56 ± 0.31 | 2.97 ± 0.43 |
| Hepatocellular water (ml) | 4.55 ± 0.40 | 6.39 ± 0.89* |

the water content of endotoxic rat livers was about 27 % more than that in the corresponding saline-treated controls.

In fact the 1.8 g increase in hepatic water content was the major component of the 2.2 g increase in total liver weight. Livers of the endotoxic rats also had significantly higher wet/dry weight ratios than those of either the normal-fed rats or the saline-treated controls (Table 1).

The dry mass, which comprises about 30 % of the total liver weight, was increased by 0.4 g in endotoxic livers (Table 1). Hepatic protein, glycogen and lipid fractions are all slightly increased in the endotoxin-treated rats compared with the saline-treated controls. This agrees with a number of other studies in which a small increase (0.1–0.2 g per liver) in hepatic protein content was reported in endotoxic [3] or septic rats [2].

## Changes in intracellular and extracellular spaces

The large increase in the liver water content may result from an increase in the volume occupied by liver cells, blood or interstitial fluid, or any combination of these. Table 2 contains data on the

**Table 1 Total mass, water content and dry mass components of the liver**

Data were obtained 24 h after the injection of endotoxin (3 mg/kg, i.p.) or saline (3 ml/kg, i.p.), or at the same clock time for the untreated fed group. Values are means ± S.D. with the number of rats in parentheses. * $P < 0.05$ versus saline-treated control group; ‡ $P < 0.05$ versus normal-fed group.

| | Normal fed ($n = 5$) | Saline-treated control ($n = 8$) | Endotoxin-treated ($n = 8$) |
|---|---|---|---|
| Body weight (g) | 326 ± 17 | 325 ± 10 | 322 ± 12 |
| Total liver mass (per liver) | | | |
| Wet weight (g) | 12.07 ± 0.66 | 9.60 ± 0.72‡ | 11.78 ± 0.71* |
| Hepatosomatic index | 3.71 ± 0.71 | 2.96 ± 0.26 | 3.66 ± 0.18* |
| Water (g) | 8.53 ± 0.48 | 6.74 ± 0.54‡ | 8.54 ± 0.61* |
| (%) | 70.34 ± 0.52 | 70.14 ± 0.76 | 72.58 ± 0.89* |
| Dry mass (g) | 3.54 ± 0.19 | 2.86 ± 0.20‡ | 3.24 ± 0.13* |
| Wet/dry ratio | 3.41 ± 0.04 | 3.36 ± 0.09 | 3.63 ± 0.13* |
| Dry mass components (per liver) | | | |
| Glycogen (g)[a] | 0.63 | 0.01 | 0.05 |
| Lipids (g) | 0.86 ± 0.09 | 0.68 ± 0.02‡ | 0.78 ± 0.10* |
| Proteins (g) | 1.62 ± 0.11 | 1.37 ± 0.11‡ | 1.54 ± 0.10* |
| DNA (mg) | 25.85 ± 1.12 | 25.51 ± 1.54 | 28.71 ± 1.55* |

[a] The glycogen data could not be obtained from the same livers as the other data since it required freeze-clamping. The glycogen content/g, obtained in a separate experiment, was multiplied by the mean of the total liver mass for each group.

**Table 3 Morphometry of hepatocytes**

Data were obtained 24 h after the injection of endotoxin (3 mg/kg, i.p.) or saline (3 ml/kg, i.p.). Values are means ± S.D. with the number of rats in parentheses. * $P < 0.05$ versus saline-treated controls.

| Hepatocyte morphometry | Saline-treated control ($n = 4$) | Endotoxin-treated ($n = 4$) |
|---|---|---|
| Perimeter ($P$) ($\mu$m) | 60.78 ± 1.50 | 63.10 ± 0.90 |
| Area ($\mu m^2$) | 246.2 ± 10.4 | 265.0 ± 10.2* |
| Volume ($V$) ($\mu m^3$) | 2977 ± 189 | 3347 ± 202* |
| Shape factor[a] | 0.81 ± 0.03 | 0.82 ± 0.00 |
| Binucleate hepatocytes (%) | 5.3 ± 2.1 | 4.8 ± 3.1 |

[a] The shape factor is the ratio of the shortest diameter over the longest diameter of an individual hepatocyte cell body (where 0 = a straight line; 1 = a perfect circle).

volume of the major components of hepatic fluid spaces. The hepatic blood content did not change after endotoxin administration. The extrahepatocellular water (which represents the total hepatic extracellular volume plus the volume occupied by blood cells) tended to increase after endotoxin treatment due to an increased volume of interstitial fluid, but this was not statistically significant (Table 2). Total hepatocellular water (the volume occupied by hepatocytes and other hepatic cells) increased by 40% in the endotoxic animals compared with the saline-treated controls.

### Changes in hepatic $K^+$ and DNA content

Cell volume is determined by the cell content of intracellular osmotically active solutes. Since about 97% of hepatic $K^+$ ions occur inside cells, we expected that an increase in the hepatocellular space would be reflected in an increase in the hepatic content of $K^+$ ions. Indeed, the total hepatic $K^+$ content in the endotoxic livers (1062 ± 86 $\mu$mol/liver, $n = 8$) was 25% higher than the livers from the saline-treated controls (852 ± 50 $\mu$mol/liver, $n = 8$) ($P < 0.001$). This increase was comparable with the increases in total liver mass (about 23%) and hepatic water content (about 27%) (Table 1). These data indicate that the expansion in the volume occupied by liver cells is primarily responsible for the increased liver mass of the endotoxic rats.

Hepatocytes account for about 65% of the cell population of the liver but over 90% of the hepatic cellular volume. The remaining non-hepatocytes (endothelial cells, Kupffer cells, fat-storing cells, etc.), represent about 35% of the cell number but, being much smaller than hepatocytes, occupy only 6–10% of the liver cellular volume [21]. Therefore, the hepatocytes are likely to be the major contributor to the substantially increased hepatocellular space. This could result from an increased number of hepatocytes and/or an increased volume of hepatocytes.

The number of hepatocytes (more strictly, the number of hepatocyte nuclei) can be estimated from the hepatic DNA content due to the constancy of DNA per nucleus in most organs and tissues of mature animals [16], provided the proportion of multinucleate hepatocytes does not change. Data in Table 1 show that the total hepatic DNA content of the endotoxic rats increased by 14% compared with the saline-treated controls. Histological examination (Table 3) revealed no significant change in the percentage of binucleate hepatocytes (about 5%). This suggests an increase in the population of liver cells after endotoxin administration but does not indicate which cell type is responsible.

**Table 4 The effect of administration of a polyclonal antiserum against TNF-$\alpha$ on the liver mass of endotoxin-treated rats**

All rats were treated with LPS (3 mg/kg, i.p.). Prior (2 h) to the injection of LPS, the rats were also pretreated with the serum IgG fraction of goat polyclonal antiserum against rat TNF-$\alpha$ (20 mg/300 g rat, i.p.) (anti-TNF-$\alpha$ group) or the control goat IgG (20 mg/300 g rat, i.p.) (normal IgG group). The median and left lateral lobes, which account for 70% of the total liver mass, were removed by the partial hepatectomy procedure 24 h after LPS injection. Before the partial hepatectomy, a portion of the liver tissue was taken from the right lateral lobe for the morphometric analysis of hepatocytes. * $P < 0.05$ versus anti-TNF-$\alpha$ + LPS group.

| | Anti-TNF-$\alpha$ + LPS ($n = 4$) | Normal IgG + LPS ($n = 4$) |
|---|---|---|
| Body weight (g) | 284 ± 8 | 281 ± 9 |
| Liver mass (g) | 6.74 ± 0.48[a] | 7.92 ± 0.50* |
| | (8.76 ± 0.62)[b] | (10.30 ± 0.64) |
| Wet/dry weight ratio | 3.33 ± 0.14 | 3.55 ± 0.06* |
| Hepatocyte morphometry | | |
| Perimeter ($P$) ($\mu$m) | 64.06 ± 2.90 | 67.10 ± 2.30 |
| Area ($\mu m^2$) | 268.2 ± 16.7 | 297.2 ± 20.6* |
| Volume ($V$) ($\mu m^3$) | 3378 ± 320 | 3966 ± 429* |

[a] The values of liver mass and hepatosomatic index are of the median and left lateral lobes.
[b] The values in parentheses are the calculated liver mass for the whole liver. All values are means ± S.D.

### Morphometric study of hepatocyte volume

Morphometric studies demonstrated increased hepatocyte volume. The mean transectional areas and the mean volumes of hepatocytes increased by 8% and 12% respectively in the endotoxic livers compared with livers from the saline-treated controls. The hepatocyte shape factors and the percentages of binucleate hepatocytes among all hepatocytes for the livers from the two groups of animals were not significantly different (Table 3).

### Mediation of LPS effect by TNF-$\alpha$

Increased production and serum levels of TNF-$\alpha$ have been observed in patients with various liver diseases with detectable hepatomegaly [22]. Long-term [23] or acute [6] administration of TNF-$\alpha$ to rats has also been reported to cause hepatic enlargement. This raises the question as to whether the effect of *E. coli* LPS on liver mass and hepatocyte volume is also mediated by the cytokine TNF-$\alpha$. We determined whether polyclonal antiserum against TNF-$\alpha$ could inhibit the effect of LPS on liver mass. Table 4 shows data from endotoxic rats pretreated with goat non-immune IgG (the control) or goat anti-(murine TNF-$\alpha$) serum. The liver mass of the animals pretreated with the control serum was 18–19% more than that in the endotoxic rats pretreated with the goat anti-(murine TNF-$\alpha$) serum. Furthermore, the increased hepatic mass in this experiment was also accompanied by a significantly larger (by 20%) hepatic water content and a higher hepatic wet/dry weight ratio. This recalls the data from animals solely treated with *E. coli* LPS (Table 1). The subsequent morphometric study found that the mean transectional area and the mean volume of hepatocyte cell bodies, in the endotoxic rats pretreated with non-immune IgG, were 11% and 17% larger, respectively, than in the endotoxic rats protected by the polyclonal anti-(murine TNF-$\alpha$) serum (Table 4). These data strongly suggest that the effect of *E. coli* LPS on the hepatic mass and hepatocyte volume is mediated by the cytokine TNF-$\alpha$.

**Table 5 Hepatic and blood L-alanine and total amino acid concentrations**

Data were obtained 24 h after the injection of endotoxin (3 mg/kg, i.p.) or saline (3 ml/kg, i.p.). Values are means ± S.D. with the number of rats in parentheses. * $P < 0.05$, versus saline-treated controls; † $P < 0.05$, versus normal-fed group at 0 h.

| | | Normal fed | Saline-treated control | Endotoxin-treated |
|---|---|---|---|---|
| Total amino acids | | | | |
| Blood | ($\mu$mol/ml) | 4.51 ± 0.50 (4) | 4.07 ± 0.23 (4) | 4.23 ± 0.27 (6) |
| Liver | ($\mu$mol/g) | 15.17 ± 2.08 (4) | 14.69 ± 2.40 (4) | 19.03 ± 2.87 (6)* |
| | ($\mu$mol/liver)[a] | 174 | 134 | 216 |
| L-Alanine | | | | |
| Blood | ($\mu$mol/ml) | 0.35 ± 0.04 (5) | 0.25 ± 0.03 (3)‡ | 0.54 ± 0.02 (3)* |
| Liver | ($\mu$mol/g) | 0.77 ± 0.10 (5) | 0.57 ± 0.37 (3) | 1.66 ± 0.65 (3)* |
| | ($\mu$mol/liver)[a] | 8.84 | 5.18 | 18.86 |

[a] The total liver mass was not available due to freeze-clamping. Therefore, the total amino acids and L-alanine per liver were calculated by multiplying the hepatic amino acid concentrations by the means for total liver mass, which were determined from the combined data (results not shown) of five experiments containing a total of 19–21 rats for each group.

### Hepatic adenine nucleotides, plasma lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios, and serum alanine aminotransferase activity

Endotoxic animals undergo dramatic changes in haemodynamics and energy metabolism. It was, therefore, important to determine whether impaired hepatic energy metabolism could underlie the observed alterations in hepatic mass and hepatocyte volume. The tissue contents of ATP, ADP and AMP in the endotoxic livers (3.35 ± 0.45, 1.18 ± 0.18 and 0.24 ± 0.07 $\mu$mol/per g of wet liver; $n = 5$) were not significantly different from those in the saline-treated controls (3.56 ± 0.21, 1.39 ± 0.08 and 0.28 ± 0.01 respectively; $n = 3$). During the 24-h time course after endotoxin administration, there was no major change in the plasma ratio of lactate/pyruvate or of β-hydroxybutyrate/acetoacetate (results not shown). This suggested that there was no significant increase in the cytoplasmic or mitochondrial NADH/NAD ratio, an index of tissue hypoxia [24]. The activities of serum alanine aminotransferase (an index of hepatic damage) in the animals treated with various LPS doses ranging from 0.03 mg/kg (22.6 ± 3.3 units/l, $n = 3$) to 3.0 mg/kg (30.0 ± 8.1 units/l, $n = 3$) were all not significantly different from that in the saline-treated controls (24.9 ± 5.0 units/l, $n = 4$). Together with the 25% increase in the liver $K^+$ content, these data do not indicate impaired energy status and tissue hypoxia, or extensive cell damage in the liver of our endotoxic animal model.

### Hepatic total amino acids and L-alanine concentrations

Mobilization of amino acids from the periphery with a concomitant increase of hepatic amino acid uptake and protein synthesis are characteristics of infections including endotoxaemia [3,23]. In our animal model the blood L-alanine levels were elevated throughout the experiment (results not shown). After 24 h of treatment, the blood L-alanine concentration in the endotoxic rats was more than double that in the saline-treated controls, and the hepatic total amino acid and hepatic L-alanine content in the endotoxic rats were 65% and 270% higher, respectively, than in the saline-treated controls (Table 5).

## DISCUSSION

In this study, we investigated, *in vivo*, the increase in liver mass and hepatocyte volume after a single i.p. administration to fasted rats of *E. coli* LPS (0127:B8) at a sublethal dose (3 mg/kg). We used fasted animals in these experiments so as to have an adequate control group since, after endotoxin administration, the animals ate very little. During fasting the liver loses weight rather rapidly and it is possible to argue that endotoxin merely prevents this normal loss of liver mass. However, this is not so, as shown by the increased wet/dry ratio and the increased DNA content in livers from endotoxin-treated fasted rats compared with those from normal-fed controls (Table 1). After 24 h, the difference between the fasted endotoxin-treated and fasted saline-treated groups amounts to about 2.6 g wet weight liver, which represents a 25% difference in the total hepatic mass for a 300 g rat (Figure 1 and Table 1). Hepatic water accounted for the great bulk of this difference. Since cells make up more than 80% of the total hepatic fluid space, we hypothesized a difference in the hepatocellular space, i.e. the total volume of the liver cells (hepatocytes and/or non-hepatocytes). The analyses of hepatic fluid spaces *in vivo* indicated indeed that less than 20% of the difference in the hepatic water content was attributable to the net change in the extrahepatocellular water content (blood plus interstitial fluid) (Table 2). An expansion by 40% of the hepatocellular water content underlay the increased total hepatic water after endotoxin administration (Table 2). The hepatic $K^+$ content, the predominant intracellular cation, also increased by 25% after endotoxin injection.

An increase in the volume of hepatocellular water could result from larger liver cells and/or more liver cells. In this study, endotoxin administration increased the hepatic DNA content by 14% (Table 1), while the proportion of binucleate hepatocytes did not change (Table 3). This suggests that there is an increase in the population of liver cells (more hepatocytes and/or more non-hepatocytes) after endotoxin treatment. However, the pathogenesis of LPS hepatotoxicity is characterized by an infiltration of white blood cells, polymorphonuclear leucocytes (PMNs) in particular, into the liver from circulation [25]. Histopathological studies have shown that the accumulation of a large number of PMNs, monocytes and Kupffer cells in sinusoids persists for at least 24 h after LPS exposure [26]. Morphological examination of the livers confirmed a substantial infiltration of PMNs, amounting to approximately 5% of total liver cells in the endotoxin-treated rats. This can make a significant contribution to the total hepatic DNA content.

Increased hepatic DNA could also occur as a result of DNA replication and cell division and it is likely that this also makes a contribution in our model, but it is less likely that this occurs in hepatocytes. Enhanced incorporation of [$^3$H]thymidine into

hepatic DNA occurs after the administration of TNF-$\alpha$, a major endogenous mediator of the effect of LPS, and this has been localized in cells other than hepatocytes, while the low level of DNA synthesis normally found in hepatocytes remains unaffected [27]. Thus, any increase in hepatic DNA content after endotoxin treatment due to DNA replication probably occurs in liver cells other than hepatocytes. Since non-hepatocytes account for 35–40 % of the liver cell population, they represent a considerable proportion of hepatic DNA. Hepatocytes, however, make up the great bulk of the intracellular space ($> 90$ %). Thus an increase in the total volume of non-hepatocytes would contribute proportionally much less to the hepatocellular water content than would a change in hepatocyte volume. It is therefore likely that the larger hepatocytes are the major liver component responsible for the marked increase in the total liver mass and hepatocellular water content. The morphometric data confirmed this hypothesis by demonstrating a significant increase (of 17 %) in hepatocyte volume in the endotoxic rats (Table 4).

The increase in hepatic mass, wet/dry weight ratio and hepatocyte volume in the endotoxin-treated rats could be prevented by the administration of polyclonal anti-TNF-$\alpha$ serum before the endotoxin injection. This suggests that TNF-$\alpha$ plays a role in mediating the effect of endotoxin on the hepatic mass. Since the serum half-life of TNF-$\alpha$ is very short (6–20 min) and circulating TNF-$\alpha$ has been reported to be cleared within 4–5 h following an endotoxin bolus [28], it is possible that some regulatory cascade initiated by TNF-$\alpha$ and possibly other cytokines are mediators of the increase in hepatic mass. In particular, endotoxin administration is followed by the appearance in plasma of TNF-$\alpha$, IL-1 and IL-6, generally in that order. Each of these cytokines is known to exercise effects on hepatic metabolism, with IL-6 playing a role as the major inducer of acute-phase protein synthesis [29]. The involvement of IL-1 in the pathology is emphasized by the demonstration that an IL-1 receptor antagonist can attenuate endotoxin-induced hypoglycaemia [30]. The production of IL-1 and IL-6, as a response to endotoxin, is reduced in animals passively immunized with anti-TNF-$\alpha$ [31]. Therefore, we do not feel that our experiments with anti-TNF-$\alpha$ permit us to conclude that direct TNF-$\alpha$ action on the liver brings about hepatic enlargement. Neither can we exclude this possibility.

Although the mechanism of hepatocyte enlargement has not been directly determined in this study, the following observations are relevant. The hepatic concentration of adenine nucleotides in the freeze-clamped endotoxic liver was not significantly different from the control group. The blood lactate/pyruvate and $\beta$-hydroxybutyrate/acetoacetate ratios, throughout the 24 h period after endotoxin injection, did not suggest tissue hypoxia (results not shown). Furthermore, there was no significant increase in the activity of serum alanine aminotransferase, an index of hepatic damage. Together with the 25 % increase in the liver potassium content, these data argue against the proposition that the increased hepatocyte volume could be due to cell swelling as a result of an inhibition of $Na^+/K^+$-ATPase. Such an inhibition could occur as a result of tissue anoxia or impaired oxidative phosphorylation. It should be noted that we have recently demonstrated that mitochondria isolated from animals treated with LPS are well-coupled [32].

It is possible that an increased amino acid uptake by hepatocytes could be responsible for the hepatocyte enlargement. The hepatic contents of total amino acids and L-alanine in the endotoxic rats were 65 % and 270 % higher, respectively, in the endotoxin-treated rats than in the controls (Table 5). The $Na^+$ co-transport of amino acids, together with the exchange of $K^+$ for $Na^+$ via $Na^+/K^+$-ATPase, has been shown to result in an intracellular accumulation of $K^+$ and amino acids and enlargement of hepatocytes in the isolated perfused liver [1]. In addition, the hepatic uptake of amino acids is not only enhanced by the increased availability of amino acids in the extracellular environment [33], but also stimulated by the cytokine TNF-$\alpha$ and glucagon, two important hormonal mediators during endotoxaemia. TNF-$\alpha$ has been shown to exhibit a permissive effect in enhancing the stimulation of amino acid transport by glucagon [34]. The marked increase in $Na^+$-dependent amino acid transport by TNF-$\alpha$ *in vivo* is, in part, mediated by glucocorticoids [35]. Alanine transport in hepatic membrane vesicles has also been reported to be stimulated in endotoxin-treated rats [36]. Thus, it is possible that the enlargement of hepatocytes after endotoxin administration is due to the enhanced hepatic uptake of amino acids, especially of L-alanine, by the activation of $Na^+$-dependent systems in the liver.

The relevance of the hepatocyte enlargement to the regulation of hepatic metabolism is of interest. Häussinger and co-workers have extensively investigated the relationship between cell volume, or hydration state, and protein metabolism [1]. They postulate that decreased cellular hydration in liver and skeletal muscle brings about a protein catabolic state in a variety of pathological conditions [37]. In contrast, insulin has been shown, in the isolated perfused liver at physiological concentrations, to affect the activity of volume-regulatory transport systems in the hepatocyte membrane, which secondarily result in a cell volume increase [1]. The insulin-induced increase in hepatocyte volume has been hypothesized to function as an 'anabolic signal' effecting the inhibition of glycogenolysis, glycolysis, proteolysis, and the stimulation of glycogen synthesis, amino acid uptake and protein synthesis [1]. Agius et al. have emphasized the role of intracellular $K^+$ in determining hepatocyte volume during the fasted-to-fed transition in rat liver [38]. Baquet et al. have shown, *in vitro*, that stimulation of glycogen synthase a and acetyl-CoA carboxylase, the key enzymes in glycogen synthesis and lipogenesis, by amino acids, results from an increase in hepatocyte volume [39]. Recent results from Häussinger's group show that the hepatic hydration state can affect the expression of phosphoenolpyruvate carboxykinase (PEPCK) mRNA [40]. In particular, exposure of isolated perfused liver or of hepatoma cells, for a few hours, to hypoosmotic conditions led to a virtual disappearance in PEPCK mRNA levels, whereas hyperosmotic conditions markedly increased them.

Many of the metabolic changes mentioned above also occur in our animal model. At 24 h, the endotoxin-treated rats did exhibit increased hepatic content of glycogen, lipids, protein and free amino acids compared with the saline-treated controls (Tables 1 and 5). In fact, with respect to glycogen metabolism, we have observed a rapid fall in hepatic glycogen, to very low levels, within 1 h of the endotoxin injection, which was followed by a slow accumulation over the next 23 h with the result that glycogen levels became greater than those in the saline-treated controls (results not shown). This net synthesis of glycogen in a fasted animal is very unusual. It is also known that endotoxin, mediated by cytokines, may lead to increased lipogenesis and decreased ketogenesis in the liver [41,42]. The incorporation of $^3H$ from $^3H_2O$ into hepatic lipids increased by 80 % at 24 h compared with the saline-treated control rats (results not shown). The plasma level of ketone bodies was depressed markedly over 24 h in the endotoxin-treated rats (results not shown) while the plasma long-chain fatty acids were elevated during 9 to 12 h (results not shown). The correlation of increased hepatocyte volume with the inhibition of hepatic ketogenesis has been suggested but remains to be further investigated [1]. Under a similar endotoxin protocol, increased hepatic protein content

was reported to occur concurrently with a stimulation of hepatic protein synthesis and with a substantially elevated insulin level in plasma [3]. Much of this protein synthesis represents the well-known increased synthesis and release of the acute-phase proteins from the liver which occur in the face of marked protein catabolism in muscle [3]. Finally, it is well established that endotoxin treatment leads to hypoglycaemia and impaired gluconeogenesis, an effect that is attributable to decreased flux through PEPCK [43,44]. In addition, endotoxin adminstration to rats decreases PEPCK synthesis, an effect that occurs at the level of transcription, and this decrease can be prevented by passive immunization with polyclonal antibodies to TNF-$\alpha$ [31].

It is, therefore, tempting to speculate that the enlargement or increased cellular hydration of hepatocytes after endotoxin administration could be a physiological mechanism that facilitates the metabolic alterations that are known to occur in endotoxaemia. Further experiments are required to test this speculation.

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## REFERENCES

1 Häussinger, D. and Lang, F. (1990) Biochim. Biophys. Acta **1071**, 331–350
2 Pedersen, P., Seeman, T. and Hasselgren, P. (1986) Acta Chir. Scand. **152**, 29–34
3 Jepson, M. M., Pell, J. M., Bates, P. C. and Millward, D. J. (1986) Biochem. J. **235**, 329–336
4 Wusteman, M., Wight, D. G. D. and Elia, M. (1990) Am. J. Physiol. **259**, E763–E769
5 Vary, T. C. and Kimball, S. R. (1992) Am. J. Physiol. **262**, C445–C452
6 Feingold, K. R. and Grunfeld, C. (1987) J. Clin. Invest. **80**, 184–190
7 Akerman, P., Cote, P., Yang, S. Q., McClain, C., Nelson, S., Bagby, G. J. and Diehl, A. M. (1992) Am. J. Physiol. **263**, G579–G585
8 Evans, R. D., Argilés, J. M. and Williamson, D. H. (1989) Clin. Sci. **77**, 357–364
9 Patton, J. S., Peters, P. M., McCabe, J., Crase, D., Hansen, S., Chen, A. B. and Liggitt, D. (1987) J. Clin. Invest. **80**, 1587–1596
10 Waynforth, H. B. (1979) Experimental and Surgical Technique in the Rat, pp. 136–142, Academic Press, London
11 Karnovsky, M. J. (1965) J. Cell Biol. **27**, 137A–138A
12 Hyam, P. L. (1981) Can. J. Med. Tech. **48**, 255–260
13 Brosnan, J. T., Man, K.-C., Hall, D., Colbourne, S. A. and Brosnan, M. E. (1983) Am. J. Physiol. **244**, E151–E158
14 Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) J. Biol. Chem. **226**, 497–509
15 Bergstrom, J. P., Larsson, J., Nordström, H., Vinnars, E., Askanazi, J., Elwyn, D. H., Kinney, J. M. and Fürst, P. J. (1987) Acta Chir. Scand. **153**, 261–266
16 Enesco, M. and Leblond, C. P. (1962) J. Embryol. Exp. Morphol. **10**, 530–562
17 Burton, K. (1956) Biochem. J. **62**, 315–323
18 Hems, D. A. and Brosnan, J. T. (1970) Biochem. J. **120**, 105–111
19 Bergmeyer, H. U. and Bernt, E. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), vol. II, pp. 752–758, Academic Press, New York
20 Oser, B. L. and Hawk, P. B. (1965) in Hawk's Physiological Chemistry, 14th edn., pp. 1095–1097, McGraw-Hill, New York
21 Blouin, A., Bolender, R. P. and Weibel, E. R. (1977) J. Cell Biol. **72**, 441–455
22 Bird, G. L. A., Sheron, N., Goka, A. K. J., Alexander, G. J. and Williams, R. S. (1990) Ann. Intern. Med. **112**, 917–921
23 Fong, Y., Morano, M. A., Moldawer, L. L., Wei, H., Calvano, S., Kenney, J. S., Allison, A. C., Cerami, A., Shires, T. and Lowry, S. F. (1990) J. Clin. Invest. **85**, 1896–1904
24 Brosnan, J. T., Krebs, H. A. and Williamson, D. H. (1970) Biochem. J. **117**, 91–96
25 Hewett, J. A. and Roth, R. A. (1993) Pharmacol. Rev. **45**, 382–411
26 Levy, M. and Ruebner, B. H. (1967) Am. J. Pathol. **52**, 477–502
27 Feingold, K. R., Baker, M. E., Johnes, A. L. and Grunfeld, C. (1991) Hepatology **13**, 773–779
28 Tracey, K. J., Lowry, S. F. and Cerami, A. (1988) in Bacterial Endotoxins (Levin, J., Buller, H. R., ten Cate, J. W., van Deventer, S. J. H. and Sturk, A., eds.), 2nd edn., pp. 77–88, Alan R. Liss, New York
29 Heinrich, P. C., Castell, J. V. and Andus, T. (1990) Biochem. J. **265**, 621–636
30 Vogel, S. N., Henricson, B. E. and Neta, R. (1991) Infect. Immun. **59**, 2494–2498
31 Hill, M. R. and McCallum, R. E. (1992) Infect. Immun. **60**, 4040–4050
32 Ewart, H. S., Qian, D. and Brosnan, J. T. (1995) J. Surg. Res. **59**, 245–249
33 Collarini, E. J. and Oxender, D. L. (1987) Annu. Rev. Nutr. **7**, 75–90
34 Warren, R. S., Donner, D. B., Starnes, J. F., Jr. and Brennan, M. F. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 8619–8622
35 Pacitti, A. J., Inoue, Y. and Souba, W. W. (1993) J. Clin. Invest. **91**, 474–483
36 Pacitti, A. J., Augsten, T. R. and Souba, W. W. (1991) J. Surg. Res. **51**, 46–53
37 Häussinger, D., Roth, E., Lang, F. and Gerok, W. (1993) Lancet **341**, 1330–1332
38 Agius, L., Peak, M. and Al-Habori, M. (1991) Biochem. J. **276**, 843–845
39 Baquet, A., Maisin, L. and Hue, L. (1991) Biochem. J. **278**, 887–890
40 Newsome, W. P., Warskulat, U., Wettstein, M., Stoll, B., Gerok, W. and Häussinger, D. (1994) Biochem. J. **304**, 555–560
41 Beylot, M., Vidal, H., Mithieux, G., Odeon, M. and Martin, C. (1992) Am. J. Physiol. **263**, E897–902
42 Memon, R. A., Feingold, K. R., Doerrler, W., Adi, S., Dinalello, C. A. and Grunfeld, C. (1992) Am. J. Physiol. **263**, E301–309
43 Jones, C. G. and Titheradge, M. A. (1993) Biochem. J. **289**, 169–172
44 Horton, R. A., Knowles, R. G. and Titheradge, M. A. (1994) Biochem. Biophys. Res. Commun. **204**, 659–665

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