Up-regulation of nitric oxide production by interferon-γ in cultured peritoneal macrophages from patients with cirrhosis

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ABSTRACT
We previously described a long-lasting overproduction of nitric oxide (NO) in cirrhotic patients with spontaneous bacterial peritonitis. The aim of the present study was to investigate the presence of the inducible NO pathway in peritoneal macrophages. Ascitic fluids were collected from 29 patients with cirrhosis, aged between 35 and 82 years. Peritoneal macrophages were isolated and cultured in the presence or absence of 1 μg/ml lipopolysaccharide and/or 500 units/ml interferon-γ (IFN-γ) for 6 days. NO production was measured as nitrate + nitrite (NOx), inducible NO synthase (iNOS) protein expression was analysed by immunocytochemistry and Western blot analysis using a specific anti-(human iNOS) antibody, and the catalytic activity of NOS was revealed by cytochemical staining for NADPH-dependent diaphorase. Cultured macrophages spontaneously released small amounts of NOx [median (10–90th percentile) of 18 separate experiments: 3.3 (0–8) μmol/l]. Addition of lipopolysaccharide alone or in combination with IFN-γ to the culture medium did not change the levels of NOx, while IFN-γ alone dramatically increased NO production [13.4 (3.5–28.3) μmol/l; P < 0.001]. Macrophages were stimulated by IFN-γ to a greater extent in patients with recent spontaneous bacterial peritonitis (n = 13) than in those in a stable clinical condition (n = 18) [19.8 (10.5–30.1) and 10.0 (3.2–14.5) μmol/l respectively; P < 0.001]. Macrophages freshly isolated or stimulated with IFN-γ expressed iNOS protein, as shown by Western blot and immunocytochemical analysis, and stained for NADPH diaphorase. Our findings demonstrate the presence of iNOS protein in peritoneal macrophages from cirrhotic patients. The role of IFN-γ appears to be a determinant for the up-regulation of NO production, particularly under conditions of infection. Therefore peritoneal macrophages producing large amounts of NO at the site of infection may contribute to maintaining splanchnic vasodilation in these patients.

INTRODUCTION
Nitric oxide (NO) is a short-lived free radical synthesized from l-arginine by the enzyme NO synthase (NOS), and it is an important mediator of a number of processes, including neurotransmission, vascular smooth muscle cell relaxation, and anti-tumour and anti-microbial activity. There are three related isoforms of NOS that are encoded by separate genes: neuronal NOS from the brain, inducible NOS (iNOS), first isolated from murine

Key words: diaphorase activity, immunoperoxidase cytochemistry, inducible NO synthase, protein expression, Western blot analysis.

Abbreviations: IFN-γ, interferon-γ; IL-1, interleukin-1; LPS, lipopolysaccharide; l-NMMA, N-monomethyl-l-arginine; (i)NOS, (inducible) nitric oxide synthase; NOx, nitrate + nitrite; TNF-α, tumour necrosis factor-α; SBP, spontaneous bacterial peritonitis.

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macrophages, and endothelial NOS, which was first described in endothelial cells. A substantial amount of information has been reported concerning the mechanisms by which pro-inflammatory cytokines, such as interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1), and/or bacterial products, such as lipopolysaccharide (LPS), up-regulate iNOS in rodent macrophages, leading to the production of large amounts of NO for prolonged periods of time [1]. Although human hepatocytes and chondrocytes can express iNOS mRNA and produce high levels of NO upon treatment with various cytokines and LPS [2,3], the ability of human mononuclear phagocytes to express iNOS has been long contested, and less is known about the regulation of iNOS in human monocytes/macrophages [4,5]. Increased production of NO has repeatedly been observed in patients with cirrhosis, particularly when the disease is decompensated, and has been hypothesized to play a role in the pathogenesis of peripheral vasodilation and hyperdynamic syndrome [6–11]. Recently we described a long-lasting overproduction of NO in terms of increased nitrate levels in the serum and ascitic fluid of cirrhotic patients with spontaneous bacterial peritonitis (SBP) [12]. Our findings argued in favour of a local peritoneal source of NO. Based on these data, we speculated that peritoneal macrophages might contribute to producing large amounts of NO at the site of infection.

In order to address this question, we have focused our interest on NO production and iNOS protein expression in vitro by cultured peritoneal macrophages from the ascitic fluid of cirrhotic patients without infection or with SBP. The regulation of NO production by IFN-γ and/or LPS was investigated. Since molecular and biochemical studies have confirmed that the catalytic activity of NOS can be revealed by reduction of a tetrazolium dye in the presence of reduced NADPH, cytochemical staining for NADPH-dependent diaphorase was also used as an additional independent approach to detect NOS [13,14].

**METHODS**

**Patients**

A total of 29 patients with alcoholic liver cirrhosis and ascites (20 men and nine women), aged between 35 and 82 years, who were admitted to our unit for liver diseases were monitored during their hospitalization. All the patients had a history of alcoholic liver disease and had had complications before admission to the unit (encephalopathy, refractory ascites, gastro-intestinal bleeding, infections, etc.). The diagnosis of liver cirrhosis was made from usual biological, clinical and endoscopic findings. The degree of liver failure was scored according to the Child–Pugh classification [15]. The patients were not being treated with corticosteroids for alcoholic hepatitis or with nitrate-containing medications at the time of the study. Neoplastic diseases precluded participation in the study. Informed consent was given by patients, and the study was carried out according to the guidelines of the local Ethics Committee.

Patients were divided into two groups. Group 1 included 13 patients with SBP, as defined by a polymorphonuclear cell count in ascitic fluid higher than 250/mm³ and/or a positive culture associated with clinical signs of sepsis (fever, hypotension, encephalopathy, diarrhoea, abdominal pain, etc.). Of the 13 cases of SBP, cultures were positive in nine cases; Gram-positive (*Enterococcus faecalis*) and Gram-negative (*Escherichia coli, Enterobacter cloacae, Citrobacter freundii, Pseudomonas sp.*) bacteria were isolated in three and six cases respectively. Group 2 included 16 patients in a stable clinical condition. The characteristics of the patients are shown in Table 1.

**Collection of biological samples**

Sera and ascitic fluids were obtained for routine survey of cirrhotic patients. Biological follow-up of patients with severe septic complications included biochemical and coagulation tests, and paracentesis for cytological examination and culture of ascitic fluid or for treatment of refractory ascites. In the case of patients from group 1, ascitic fluids destined for cell culture were collected 9 ± 2 days after biological diagnosis of SBP was confirmed.

**Cell preparation**

Ascitic fluid (approx. 1500 ml) was collected into sodium citrate. Haemorrhagic fluids were discarded to avoid contamination with circulating monocytes. Peritoneal cells were harvested by centrifugation at 1000 g for 45 min at room temperature. The pellet was resuspended in 10 ml of Hanks balanced salt solution (Gibco) and was then layered over 5 ml of Ficoll Paque (Pharmacia). After centrifugation at 400 g for 30 min at 15 °C, mononuclear cells were recovered from the gradient interface, washed twice with Hanks balanced salt solution, and finally adjusted to 10⁵ cells/ml in a nitrate/nitrite-free culture medium (Gibco) containing 1 mM L-arginine (Sigma), 1 mM L-glutamine (Gibco) and 50 µg/ml gentamicin, supplemented with 5% (v/v) heated fetal calf serum (Gibco) guaranteed to contain less than 20 pg/ml endotoxin and containing 25 µmol/l nitrate. The cell suspension was plated into 24-well tissue culture plates (Costar) at 500 µl per well (each well contained a 14-mm diameter coverslip), and was allowed to adhere for 3 h at 37 °C in a humidified 5% CO₂ atmosphere. Then the non-adherent cells were removed and the remaining cells were incubated in duplicate in 500 µl/well fresh medium containing various activators and/or inhibitors: 500
Nitrate and nitrite measurements
The nitrate concentration was measured by an enzymic method using nitrate reductase from Aspergillus species, as previously described [16]. Briefly, 100 μl portions of supernatant were equilibrated with 300 μl of 100 mmol/l potassium phosphate buffer (pH 7.5), 50 μl of 0.2 mmol/l FAD and 10 μl of 12 mmol/l β-NADPH, in the presence or absence of 40 μl of 500 units/l nitrate reductase. The decrease in absorbance at 340 nm was measured, and the nitrate concentration was calculated using a theoretical factor [16].

The nitrite concentration was measured using the Griess reaction. Briefly, 50 μl portions were mixed with 300 μl of 1% sulphamic acid and 300 μl of 0.1% N-1-naphthyl ethylenediamine. The absorbance at 540 nm was read after 10 min. Nitrite concentrations were calculated from a calibration curve prepared with sodium nitrite standards.

The conditioned medium supplemented with fetal calf serum contained easily detectable levels of nitrate and nitrite. Therefore nitrate and nitrite concentrations from each cell culture well were corrected for values of blanks given by the corresponding cell-free medium.

Immunocytochemical study of iNOS protein
Immediately after adhesion, cells were processed for analysis of iNOS protein using a rabbit IgG raised against an epitope corresponding to amino acids 1135–1153 mapping at the C-terminus of human iNOS; this antibody is not cross-reactive with neuronal or endothelial constitutive NOS (Santa Cruz Biotechnology, Tebu, France). The procedure was as follows. Cells were fixed with cold acetone for 2 min, then rinsed with PBS. They were then incubated with 300 μl of polyclonal rabbit anti-iNOS IgG diluted in PBS (1:200) for 60 min at 37 °C. After washing with PBS, cells were incubated with peroxidase-labelled anti-rabbit IgG (Santa Cruz Biotechnology) diluted in PBS (1:500) for 60 min at 37 °C. Staining was performed with diamobenzidine solution (0.05% diaminobenzidine, 50 mM Tris/HCl, pH 7.4, 0.01% freshly prepared H2O2) for 10 min at room temperature, and counterstaining was with Mayer’s haematoxylin. Staining was absent when no primary antibody was used, as a control for non-specific labelling by components of the detection system.

NADPH diaphorase staining
To detect diaphorase activity, cells plated on coverslips in 24-well tissue culture plates were incubated in 100 mM Tris/HCl, pH 7.5, containing 1 mM NADPH and 0.3 mM Nitroblue Tetr lazolium for 1 h at 37 °C [17,18]. As a negative control, cells were incubated in the same staining solution in the absence of NADPH.

Western blot analysis of iNOS protein
Macrophages obtained after adhesion, or after a 6-day culture in the presence of IFN-γ, were washed twice in ice-cold PBS and treated with 10 mM Tris buffer, pH 7.5, added with various protease inhibitors (5 mM EDTA, 1 mM PMSF, 5 μg/ml pepstatin, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 mM benzamidine) for 10 min on ice. The cells were sonicated on ice (3 × 10 s).

Equal amounts (30 μg) of each cell lysate were loaded and electrophoresed on an 7.5% (w/v)-polyacrylamide gel in the presence of SDS (Mini Protein II; Bio-Rad Laboratories). Proteins were then transferred to a nitrocellulose membrane and probed with rabbit anti-iNOS IgG (diluted 1:500). After washing, the membrane was

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**Table 1  Characteristics of the patients**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Sex (male/female)</th>
<th>Age (years)</th>
<th>Child classification (A/B/C)</th>
<th>Pugh score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13</td>
<td>10/3</td>
<td>54 (41–67)</td>
<td>0/4/9</td>
<td>13 (9–14)</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>10/6</td>
<td>49 (41–73)</td>
<td>0/9/7</td>
<td>9 (8–13)</td>
</tr>
</tbody>
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units/ml recombinant human IFN-γ (R&D Systems), 1 μg/ml LPS from Escherichia coli O55:B5 (Sigma) and 1 mM N-monomethyl-l-arginine (l-NMMA) (Sigma). Cell-free media from each well and supplemented with the same components were incubated simultaneously. The medium was renewed after 3 days of incubation. The supernatants removed on day 3 and day 6 were immediately centrifuged at 10,000 g and assayed for nitrate and nitrite, the combined measurement of which appears to account fully for NO formed in oxygen-containing media. May–Grunwald–Giemsa and esterase staining of slides after removal of supernatants enabled cytochemical characterization after the adhesion step and after a 6-day incubation. By these criteria, more than 80% of the adherent cells were macrophages.
exposed to peroxidase-labelled anti-(rabbit IgG) (diluted 1:1000). The NOS bands were revealed by enhanced chemiluminescence and detected with photographic film.

**Statistical analysis**
Results are expressed as medians with interquantile range, along with the 10th and 90th percentiles. The Mann–Whitney test was used for comparison of data between the two groups of patients. Within-subject comparisons used the Wilcoxon test. A $P$ value of $\leq 0.05$ was considered to represent a significant difference.

**RESULTS**
We have studied NO production by cultured peritoneal macrophages isolated from the ascitic fluid of patients with cirrhosis. Spontaneous release of NO by cultured cells was low, leading to the appearance of nitrate and nitrite in proportions of 9:1 (Figure 1). In addition, the responsiveness of isolated cells to IFN-γ, LPS or a combination of the two components was assessed over a 6-day period (Figure 1). Analysis of results from 18 individual donors (10 stable and eight infected) showed no modification of NOx levels by LPS alone or in combination with IFN-γ. In contrast, IFN-γ alone dramatically increased NO production [NOx median (10–90th percentile): control, 3.3 (0–8) μmol/l; IFN-γ, 13.4 (3.5–28.3) μmol/l; $P < 0.001$] (Figure 1). The two groups of patients as defined in the Methods section (group 1, recent infection; group 2, stable clinical condition) showed no significant difference in the basal levels of NOx produced by cells cultured in the absence of stimuli [1.6 (1.0–6.1) and 2.0 (0–5.7) μmol/l respectively] (Figure 2). In contrast, addition of IFN-γ to the culture medium increased NO production to a greater extent in cells from group 1 patients [19.8 (10.5–30.1) μmol/l; $P < 0.01$] than in those from group 2 [10.0 (3.2–14.5) μmol/l; $P < 0.02$], with the amount of NOx produced being significantly higher in the former than in the latter ($P < 0.001$) (Figure 2).

We followed the time course of serum nitrate levels in a patient suffering from a bout of SBP (Figure 3). The

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**Figure 1** NOx production by peritoneal macrophages from cirrhotic patients with ascites
Cells were cultured for 6 days with medium alone, 500 units/ml IFN-γ, 1 μg/ml LPS, or IFN-γ + LPS. Results from 18 individual patients (10 stable and eight infected) are shown as medians with interquantile range (box), along with the 10th and 90th percentile range (vertical lines). Using the Wilcoxon test, NOx concentrations differed significantly only between the cells cultured with medium and those cultured with IFN-γ alone ($P < 0.001$).

**Figure 2** NOx production by cultured peritoneal macrophages from cirrhotic patients in a stable condition ($n = 18$ separate experiments) or with SBP ($n = 13$)
Cells were cultured for 6 days with medium alone or with 500 units/ml IFN-γ. Results are shown as medians with interquantile range (box), along with the 10th and 90th percentile range (vertical lines). Using the Mann–Whitney test, IFN-γ-induced NOx production differed significantly between the two groups of patients ($P < 0.001$).

**Figure 3** Evolution of serum nitrate concentrations and production of NOx by peritoneal macrophages from a cirrhotic patient who had a SBP diagnosed on day 1, with paracentesis on days 7 and 21
Cells isolated on days 7 and 21 were cultured in triplicate for 6 days with medium alone, 500 units/ml IFN-γ, 1 μg/ml LPS or IFN-γ + LPS. Results are means ± S.E.M.
403

Figure 4  Dose-dependent effect of IFN-γ on NO₃ production by peritoneal macrophages from cirrhotic patients
Cells were incubated with medium alone or with IFN-γ (100 or 500 units/ml). Values are medians with interquartile range (boxes), along with the 10th and 90th percentile range (vertical lines), for six separate experiments.

Figure 5  Effects of L-NMMA on NO₃ production by peritoneal macrophages from cirrhotic patients
Cells were incubated with medium alone, 500 units/ml IFN-γ or 500 units/ml IFN-γ plus 1 mM L-NMMA. Values are the medians with interquartile range (box), along with the 10th and 90th percentile range (vertical lines), for nine separate experiments (six stable and three infected patients).

The highest nitrate level recorded was 101 μmol/l on day 17. During this period, the patient underwent paracentesis on day 7 (serum nitrate 41 μmol/l) and on day 21 (serum nitrate 75 μmol/l), from which cell cultures were obtained. NO₃ production under all culture conditions, i.e. medium alone, IFN-γ alone, LPS alone and IFN-γ + LPS, was greater in macrophages isolated on day 7 than in those isolated on day 21 (Figure 3). The highest amount of NO₃ produced by these macrophages was 37 ± 0.5 μmol/l, following IFN-γ stimulation.

In addition, cells were incubated with two concentrations of IFN-γ (100 and 500 units/ml) (Figure 4). Compared with values from cells cultured with medium alone, no significant stimulatory effect was observed at 100 units/ml IFN-γ [medians from six separate experiments: control, 1.6 (1.0–3.8) μmol/l; IFN-γ, 1.3 (1.0–10.4) μmol/l]. An increase in NO₃ production became significant only when the IFN-γ concentration was raised to 500 units/ml [14.5 (10.0–21.7) μmol/l; P < 0.05]. The specificity of this synthesis was studied by adding 1 mM L-NMMA to the culture medium initially supplemented with 1 mM l-arginine. The presence of this competitive inhibitor resulted in a decrease of about 50% in the ability of cells to produce NO (Figure 5).

Immunocytochemical studies with rabbit IgG against human iNOS were performed immediately after the cell adhesion procedure, and revealed protein immunoreactivity in macrophages from infected patients as well as in those from patients in a stable clinical condition (Figure 6A). No positive staining was seen when no primary antibody was used (Figure 6B). Moreover, the NOS-positive cells stained for NADPH diaphorase, showing the co-localization of these two proteins (Figure 6C). In the absence of NADPH, no staining was observed.
In addition, Western-blot analysis with an antibody to human iNOS was performed on lysates from peritoneal macrophages (Figure 7). The protein appeared to be expressed more strongly in macrophages freshly isolated from infected patients than in cells from patients in a stable condition. However, after a 6-day incubation in the absence of stimulator, the protein was no longer detected, whereas it was highly expressed in IFN-γ-stimulated cells, particularly those from infected patients.

**DISCUSSION**

While it has long been established that cultured murine peritoneal macrophages can produce high levels of NO (measured as nitrite) after stimulation with LPS and various cytokines, including IFN-γ, IL-1 and TNF-α, the presence of an analogous high-output nitrite-producing system in human monocytes or macrophages has been contested [4,5]. In the present study, peritoneal macrophages from cirrhotic patients spontaneously released a small amount of NO and were not stimulated by LPS. This is in line with data from Morales-Ruiz et al. [19], who observed a lack of sensitivity to LPS of peritoneal macrophages from cirrhotic rats. Moreover, previous exposure to endotoxin in vivo was shown to reduce the subsequent ability of LPS to induce iNOS in macrophages in vitro [20]. This is also in agreement with the observation that pre-exposure of peritoneal macrophages in vitro to low concentrations of LPS suppresses the induction of iNOS when IFN-γ is added subsequently. This suppression can be explained by a decreased content of iNOS protein and mRNA by 24–48 h after pre-treatment with LPS [21,22]. In contrast, peritoneal macrophages from cirrhotic patients responded to stimulation by IFN-γ in the culture medium by increasing NO\textsubscript{x} levels by 5–10-fold. The NO produced was measured mainly as nitrate. In accordance with our findings is the observation of Weinberg et al. [23], who found low levels of nitrate in human blood monocytes cultured with several different combinations of cytokines, growth factors and microbial stimulants, whereas there was a small (although statistically significant) increase in NOS activity and NO production after treatment with LPS + IFN-γ in human peritoneal macrophages. Moreover, we observed that macrophages from recently infected patients appeared to be more responsive to IFN-γ, since addition of this cytokine increased levels of NO production to a greater extent in cell cultures from these patients than in cultures from patients in a stable clinical condition. This suggests that macrophages from infected patients were already triggered by stimulation in vivo, resulting in accelerated NO production in vitro in response to additional IFN-γ. On the other hand, blood monocytes from patients with rheumatoid arthritis were shown to increase their levels of nitrite/nitrate and NOS activity when cultured in vitro in the presence of LPS, IFN-γ or a combination of the two [24]. Thus it seems that human monocytes/macrophages are able to produce appreciable amounts of NO in selective pathological situations, and therefore may contribute to NO over-production in vivo, measured as nitrate in plasma [5].

The presence of the inducible NO pathway in peritoneal macrophages from cirrhotic patients was confirmed by Western-blot and cytochemical analysis of iNOS protein using a specific anti-(human iNOS) antibody. It is noteworthy that freshly isolated macrophages expressed iNOS protein, but lost this capacity with time in culture, unless they were stimulated by IFN-γ. To test for enzymic activity in peritoneal cells, we used NADPH diaphorase cytochemistry. Cells positive for iNOS protein, but lost this capacity with time in culture, unless they were stimulated by IFN-γ. To test for enzymic activity in peritoneal cells, we used NADPH diaphorase cytochemistry. Cells positive for iNOS protein, but lost this capacity with time in culture, unless they were stimulated by IFN-γ.
by chondrocytes and synoviocytes in inflammatory joints of arthritides [25]. Human hepatocytes can be induced to produce NO after stimulation with a mixture of IL-1β, TNF-α, IFN-γ and endotoxin [2]. Moreover, the promoter region for the human hepatocyte iNOS gene has been partially analysed, revealing a TATA box, consensus sequences for three IFN-response elements and a nuclear factor-xB site [26]. Therefore further analysis of the promoter region of the human monococyte/macrophage iNOS gene should reveal important information regarding most effective stimuli for activation in order to obtain a high level of transcription and subsequent NOS and NO production.

The significance of NO synthesis in rodent macrophages has been well established. One of the most relevant biological functions of NO produced by iNOS, other than vasodilation, is its powerful microbicidal activity in vitro against Mycobacterium avium, Trypanosoma cruzi and Leishmania major [27–29]. Inhibition of iNOS in experimental cirrhosis has been shown to impair the host defence system, resulting in bacterial peritonitis [19]. Therefore iNOS induction in the macrophages of cirrhotic rats has been hypothesized to represent a host defence response to prevent bacterial infection. However, it is not certain whether an analogy can be made with human cirrhotic patients. Indeed, it still remains to be elucidated whether the consequences of iNOS expression are predominantly beneficial or potentially harmful.

In conclusion, our findings demonstrate the presence of iNOS protein in peritoneal macrophages from patients with cirrhosis. IFN-γ appears to play a key role in the up-regulation of NO production, particularly under conditions of infection. Therefore peritoneal macrophages producing large amounts of NO at the site of infection may contribute to maintaining splanchnic vasodilation, and thus worsen the hyperkinetic state in these patients.

REFERENCES


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