The Detection of Bacterial DNA in Blood of Rats With CCl_4-Induced Cirrhosis With Ascites Represents Episodes of Bacterial Translocation

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Bacterial DNA (bactDNA) is present in blood and ascitic fluid (AF) in a third of patients with cirrhosis and ascites, but whether this phenomenon represents episodes of bacterial translocation (BT), strictly considered when culture of mesenteric lymph nodes (MLNs) are positive, remains unknown. This study assessed the relationship between bactDNA detection in biological fluids and MLNs and went on to investigate the local and systemic inflammatory status according to its presence. Cirrhosis was induced in rats by ingestion of CCL_4. A subgroup of five animals with cirrhosis received norfloxacin (5 mg/kg/day) for 7 days. MLNs and ascitic and pleural fluids were collected at laparotomy and cultured; samples were collected for identification of bactDNA and measurement of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and nitric oxide (NO). BactDNA was detected in MLNs in 12 of 19 animals (63.1%), corresponding in seven cases to culture-positive MLNs, and in five to culture-negative MLNs. BactDNA was detected in biological fluids in 11 of 19 animals (57.9%), and in all cases the same bacteria *spp.* detected in samples was present in MLNs. BactDNA was not detected in any biological sample from animals receiving norfloxacin. Tumor necrosis factor alpha (TNF-α), IL-6, and NO were similar in culture-positive and culture-negative/bactDNA-positive samples, and significantly higher than those observed in animals with culture-negative/bactDNA-negative MLNs, animals with cirrhosis that were receiving norfloxacin, and controls. In conclusion, the presence of bactDNA in biological fluids in rats with cirrhosis constitutes a marker of BT, and it is associated with a marked inflammatory response, independent of the result of the culture. (HEPATOLOGY 2006;44:633-639.)

Bacterial translocation (BT) is considered a key event in the pathogenesis of bacterial infections in patients with advanced cirrhosis,[1] and spontaneous bacterial peritonitis is probably the most relevant infection in this setting.[2] BT is considered to be present either in patients or in animal models of cirrhosis when the culture of mesenteric lymph nodes (MLN) shows the growth of at least one bacterial species.[3] The genetic identity of bacteria isolated in ileal content, MLNs, and infected ascitic fluid (AF) in rats with experimental cirrhosis[4] has been shown, which supports the contention of a continuum among intestinal lumen, translocation to MLN, and eventual induction of spontaneous bacterial peritonitis. However, the study of BT is logically difficult in patients with cirrhosis.

We have shown that roughly 30% of patients with advanced cirrhosis and AF show the simultaneous presence in blood and AF of fragments of bacterial DNA (bactDNA), mostly from the same type of bacteria,[5] and that these fragments may last in blood during variable periods.[6] The identity of the nucleotide sequences detected in a given patient during the study period, together with the fact that bactDNA may disappear and then re-

Abbreviations: BT, bacterial translocation; MLN, mesenteric lymph nodes; AF, ascitic fluid; PCR, polymerase chain reaction; TNF-α, tumor necrosis factor alpha; IL-6, interleukin-6; NOx sum of NO metabolites, nitrite and nitrate.

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appear in consecutive serum specimens, lead us to consider that the main mechanism promoting the presence of bactDNA in blood was repeated episodes of bacterial movement through the intestinal wall, and not a clearance defect.[6]

The presence of bacteria in MLNs is mostly related to the carrying of bacteria by dendritic cells, either in viable (capable of growing in adequate culture media) or nonviable forms, and the ability to induce a certain secretion of IgA may be related to its viability.[7] Because we have previously shown that bactDNA may be detected in culture-negative samples, similar results might be obtained studying MLNs, and then different inflammatory reactions might be observed in samples containing viable or nonviable bacteria.

Two questions arise in this setting: first, it is necessary to assess whether the presence of bactDNA in blood, ascitic, or pleural fluids truly represents episodes of BT, which requires the simultaneous presence of bactDNA of a given bacteria in these biological fluids and in MLNs; and second, to investigate the local and systemic inflammatory consequences of the presence of bacteria in MLNs and biological fluids. This study was designed to explore both questions.

Materials and Methods

Induction of Cirrhosis

Male Sprague-Dawley rats were included in this study. Rats were individually caged at a constant room temperature of 21°C and exposed to a 12:12 light/dark cycle.

Cirrhosis was induced as previously described by Runyon et al.[8] Rats weighing 100 to 120 g were fed standard rodent chow (B/K) and were treated with 1.5 mmol/L phenobarbital in tap water. When the rats reached a weight of greater than 200 g, weekly doses of CCl_4 (J.T. Baker Inc., Phillipsburg, NJ) were given intragastrically using a sterile pyrogen free syringe (Artsana p.p.a., Grenclate) with an attached stainless steel animal feeding tube (Popper and Sons, New Hyde Park, NY) without anesthesia. The first dose of CCl_4 was 20 μL, and subsequent doses were adjusted based on changes in weight 48 hours after the last dose as previously reported.[1] After ascites appeared, the dose was reduced to 40 μL per week until laparotomy.

A subgroup of five rats with cirrhosis with ascites received norfloxacin by gavage for 7 days (5 mg/kg/day) before laparotomy. A second group of five healthy animals were studied as controls.

Laparotomy

Animals with induced cirrhosis were killed when ascites was evident. Laparotomy was performed under anesthesia with 10 mg/kg xylacine (Rompun, Bayer, Leverkusen, Germany) and 50 mg/kg ketamine (Ketolar, Parke-Davis, New York, NY) in strictly sterile conditions. Abdominal fur was removed with a depilatory, and the skin was sterilized with iodine. A short incision in the abdominal wall was performed, and one sample of AF was obtained for bacterial culture, and another sample was injected into rubber-sealed, pyrogen-free tube (Endo-Tube ET; Chromogenix AB, Vienna, Austria). The abdomen was then opened widely, and the remaining AF was evacuated. Samples of pleural fluid were collected for bacterial culture and also inoculated in pyrogen-free tubes for bactDNA studies. All detectable MLNs, especially from the ileo-cecal area, were aseptically dissected, removed, and then liquefied in sterile saline for bacterial culture. An aliquot of liquefied MLNs was stored into sterile conditions for bact-DNA study. Blood was collected from the cava vein and injected in pyrogen-free tubes. Rats were then killed with intravenous sodium thiopentate (Penthotal, Abbott Laboratories, Chicago, IL).

Control rats were killed following the same protocol, and MLNs and blood were cultured and stored for molecular assays.

This study was approved by the Animal Research Committee of the Institut de Recerca of Hospital de la Santa Creu i Sant Pau (Barcelona) and by the Departament de Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya (DARP). Animals received care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals.

Detection and Identification of bactDNA

DNA Isolation. Two hundred microliters serum, AF, or MLN homogenate were incubated in a lisozime-proteinase K buffer for 2 hours and applied into QIAamp Spin Columns (QIAamp DNA Mini Kit, QIAGEN, Hilden, Germany). Samples were spun down in a microcentrifuge at full speed, and DNA was finally eluted with 50 μL 70°C preheated water. The yield and purity of DNA were measured by reading A_{260} and A_{260}/A_{280} in a SmartSpec 3000 Spectrophotometer (BioRad, Hercules, CA).

DNA Amplification and Sequencing. A polymerase chain reaction (PCR) for the universal amplification of a region of the 16S rRNA gene was developed. Two microliters template were added to a reaction mix containing 10 mmol/L Tris buffer (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L of each dNTP, 50 pmoles primers 5′-AGAGTTTGATCATGGCTCAG-3′ and

5′-ACCGCGACTGCTGCTGGCAC-3′,[9] and 1.25 units BioTaq (Bioline, London, UK) to complete a final volume of 50 μL. The primers located at positions 7-27 and 531-514 (*E. coli* numbering) are universal bacterial primers that will amplify any known bacterial 16S rRNA gene. A 35-cycle PCR was run in a GeneAmp 9700 (Applied Biosystems, Foster City, CA) using the following profile: 94°C 30 seconds, 55°C 30seconds, and 72°C 60 seconds.

Total PCR reaction volume was filtered with QIAquick Spin Columns (QIAquick PCR Purification Kit, QIAGEN) to remove primers and dNTPs.

Five microliters purified products were analyzed by 2% agarose gel electrophoresis and UV visualization. As expected, a band of approximately 540 bp was obtained from different bacterial cultures corresponding to the specific amplification of the prokariotic 16S rRNA gene.

Nucleotide sequences of PCR products were determined by using the ABIPRISM Dye Terminator Cycle Sequencing v2.0 Ready Reaction Kit (Perkin Elmer, Wellesley, MA) and ABIPRISM 310 automated sequencer, according to the manufacturer's indications. The PCR fragments were sequenced with primer 5′ ACCGCGACTGCTGCTGGCAC 3′.[10] The identification of sequences was carried out by BLAST at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).[11]

Measurement of Cytokine and NOx Levels in MLNs, Serum, and AF Samples

The sum of the NO metabolites nitrite (NO_2^-) and nitrate (NO_3^-) is widely used as an index of NO generation and expressed as NOx levels. NOx levels were calculated by measuring conversion of NO_3^- to NO_2^- by the enzyme nitrate reductase using an ELISA assay (R&D Systems, Minneapolis, MN) based on the Griess reaction that absorbs visible light at 540 nm, and expressed as μmol/L. All samples were tested in duplicate, and values were corrected by running samples with culture media to assess background NOx levels. Immunoenzymometric assays for quantitative measurement of rat tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) in AF samples were performed by handling rat TNF-α and IL-6 Quantikine (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions, and both expressed as pg/mL. All samples were tested in duplicate and read at 450 nm and 490 nm in a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA).

Statistical Analysis of Experimental Data

Quantitative data are reported as mean ± SD. Categorical data are expressed as frequencies or percentages. Statistical differences between groups were analyzed using the Mann-Whitney *U* test for quantitative data and the chi-squared test for categorical data applying the Yate's correction when required. All *P* values were two-tailed. A *P* value of less than .05 indicated statistical significance. Analyses were performed with the SPSS Statistical package (SPSS Inc. Version 11.0, Chicago, IL).

Results

Thirty-five rats were included in the current study. Eleven rats (31.4%) died during the induction of cirrhosis, and 24 animals with cirrhosis developed ascites and were included in this study. Five rats with ascites were treated for 7 days with norfloxacin before laparotomy.

A series of concepts were considered to evaluate the results obtained. First, BT corresponds to culture-positive MLNs, non-BT corresponds to culture-negative MLNs, non-BT bactDNA positive corresponds to culture-negative MLNs with presence of bactDNA, and non-BT bactDNA negative corresponds to culture-negative MLNs and absence of bactDNA.

Bacterial Translocation (BT). None of the control animals and none of the animals receiving norfloxacin had a positive culture in MLNs. BT was observed in 7 of 19 animals with cirrhosis with ascites (36.8%) but in none of animals with cirrhosis that were treated with norfloxacin. Positive cultures in either MLNs or any of the studied biological fluids were present in nine cases (47%). Table 1 shows the results of cultures performed in MLNs, AF, and pleural fluid in all animals when samples were available.

Gram-positive cocci were isolated in two of seven culture-positive MLNs (29%), and gram-negative bacteria in the remaining five samples (71%). BactDNA fragments were identified in all MLNs with positive cultures, and there was a 100% sequence match with the type of bacteria identified in culture.

Non-BT and Relationship to bactDNA. BactDNA was not detected in any rat with cirrhosis with ascites receiving norfloxacin and in only one control rat (20%), corresponding to *Clostridium spp.*

BactDNA was detected in MLNs from 12 animals, seven being from culture-positive MLNs (BT) and the remaining five from culture-negative MLNs (non BT). BactDNA was detected in 5 of 12 animals with culture-negative MLNs (42%). Table 2 details the type of bacteria

Table 1 Results of Microbiological Cultures in All Samples When Available

Rat	MNa Culture	Fluid Culture	Pleural Fluid Culture
1	Neg	Neg	NA
2	Neg	*E. coli*	NA
3	*E. coli*	Neg	Neg
4	*S. aureus*	Neg	NA
5	Neg	Neg	NA
6	Neg	Neg	Neg
7	Neg	Neg	Neg
8	Neg	Neg	Neg
9	*E. coli*	Neg	*E. coli*
10	*E. coli*	Neg	*E. coli*
11	Neg	Neg	Neg
12	Neg	Neg	Neg
13	Neg	Neg	Neg
14	Neg	Neg	*E. coli*
15	*E. coli* + enterococcus	*E. coli* + enterococccus	*E. coli* + enterococcus
16	Neg	Neg	Neg
17	*aeruginosa*	Neg	Neg
18	*Enterobacteria*	*Enterobacteria*	*Enterobacteria*
19	Neg	Neg	Neg
1C	Neg	NA	NA
2C	Neg	NA	NA
3C	Neg	NA	NA
4C	Neg	NA	NA
5C	Neg	NA	NA
1-5 NOR	Neg	Neg	Neg

Neg, culture-negative sample; NA, sample not available; 1C-5C represent control animals; 1-5NOR represent animals receiving norfloxacin.

detected and identified in MLNs by means of PCR and nucleotide sequencing.

Presence of Bacteria in Biological Fluids. Six animals presented positive cultures of AF or pleural fluid, and in five cases the same bacteria species was isolated in MLNs in culture in the corresponding animal. One animal showed a positive culture of AF by *Escherichia coli* and one animal a positive culture of pleural fluid by the same bacteria species in the presence of a negative-culture MLN (Table 1).

BactDNA was detected and identified in 11 samples of serum (Table 2), corresponding to *E. coli*, *S. aureus*, *Klebsiella spp, Enterococcus,* and *Pseudomonas.* When considering these animals, bactDNA was detected and identified in 8 of 10 samples of AF (AF was not available for PCR testing in one animal) and in one of six pleural fluid samples, and among them five corresponded to culture-negative samples (55.5%). In all cases, bactDNA from the same bacteria species as identified in biological fluids was present in MLNs as either culture-positive (n = 7) or culture-negative (n = 4) samples.

The degree of correspondence of the type of bacteria growing in cultures of biological fluids with that from MLNs in a given animal was four of six (75%), because in two cases the corresponding culture of MLNs was negative (Table 1). When considering this same concept according to the presence of bactDNA in biological fluids and its simultaneous presence in MLNs in a given animal, nucleotide sequence similarity was 100%. In all cases when bactDNA was detected in serum, AF, or pleural fluid, DNA from the same bacteria species was present in MLNs, either as culture-positive (n = 7) or culture-negative (n = 4) samples (Table 2).

Cytokine and NOx Levels in MLNs, Serum, and AF According to BT Status. Table 3 shows values of TNF-α, IL-6, and NOx measured in serum and AF in all animals distributed in groups as previously defined (see previous discussion). In most cases, values observed in AF or serum were significantly higher in animals with BT (culture-positive MLNs) than in animals without BT (culture-negative MLNs). However, when values from animals without BT were distributed according to the presence or absence of bactDNA, marked differences were observed. This new separation of groups greatly reduced the variation of data, and values observed in culture-negative bactDNA-positive animals were similar to those observed in animals with presence of BT.

Table 2. Results of bactDNA Detection and spp Identification Through Nucleotide Sequencing in All Samples When Available

Rat	MLNs	Serum	Aecitic Fluid	Pleural Fluid
1	Neg	Neg	Neg	NA
2	*E. coli*	*E. coli*	*E. coli*	NA
3	*E. coli*	*E. coli*	Neg	Neg
4	*S. aureus*	*S. aureus*	*S. aureus*	NA
5	*S. aureus*	*Staphyfococcus sp.*	Neg	NA
6	*spp.*	*spp.*	*spp.*	Neg
7	Neg	Neg	Neg	Neg
8	Neg	Neg	Neg	NA
9	*E. coli*	*E. coli*	*E. coli*	NA
10	*E. coli*	*E. coli*	*E. coli*	NA
11	Neg	Neg	Neg	NA
12	Neg	Neg	NA	NA
13	Neg	Neg	Neg	Neg
14	*E. coli*	*E. coli*	*E. coli*	Neg
15	*E. coli*	*E. coli*	*E. coli*	*E. coli*
16	Neg	Neg	Neg	NA
17	*Pseudomonas*	*Pseudomonas*	NA	NA
18	*Enterobacteria*	*Enterobacteria*	*Enterobacteria*	NA
19	*E. coli*	N	Neg	NA
1C	Neg	Neg	NA	NA
2C	Neg	Neg	NA	NA
3C	Neg	Neg	NA	NA
4C	Neg	Neg	NA	NA
5C	*Clostridium spp.*	*Clostridium spp.*	NA	NA
1-5 NOR	Neg	Neg	Neg	Neg

Neg, culture negati sample NA, sample not available; 1C- 5C represent control animals; MLNs: mesenteric lymph node.

Table 3. Values of TNFα (pg/mL), IL6 (pg/mL), and NQx (μmol/mL) in AF and Serum From Experimental Groups and Controls

		BT According to Classic Definition				Non-BT	
		Control (n = 5)	Norfloxacin (n = 5)	BT (n = 7)	Non-BT (n = 12)	bactDNA(−) (n = 7)	bactDNA(+) (n = 5)
AF	TNF-α		19.48 ± 3.26*	47.36 ± 8.59†	31.80 ± 16.07	19.23 ± 3.73	49.40 ± 5.07‡
	IL6		10.89 ± 1.68†	22.71 ± 5.79†§	15.90 ± 6.37	11.81 ± 2.57	21.63 ± 5.61‡
	NOx		19.93 ± 5.22*	56.55 ± 3.67†§	29.84 ± 16.18	17.45 ± 3.81	47.18 ± 7.30‡
Serum	TNF-α	5.25 ± 0.49	16.97 ± 4.03*	44.08 ± 7.97†§	26.14 ± 14.20	14.93 ± 1.87	41.82 ± 4.58‡
	IL6	5.01 ± 0.02	11.91 ± 3.21*	25.01 ± 6.76†§	15.30 ± 6.42	10.66 ± 2.49	21.80 ± 3.66‡
	NOx	13.72 ± 2.39	18.63 ± 3.56*	65.83 ± 4.64†§	34.55 ± 18.19	19.61 ± 2.13	55.46 ± 8.27‡

Abbreviations: BT, bacterial translocation (culture-positive mesenteric lymph nodes); Non-BT, absence of bacterial translocation (culture-negative mesenteric lymph nodes); bactDNA(+), presence of bactDNA; bactDNA(−), absence of bactDNA; TNF-α, tumor necrosis factor alpha; NOx, sum of nitric oxide metabolites, nitrite and nitrate; IL6, interleukin 6.

*$P < .05$ Norfloxacin group vs. BT and vs. non-BT bactDNA(+).

†$P < .05$ BT vs. non-BT bactDNA(−).

‡$P < .05$ Non-BT bactDNA(+) vs. Non-BT bactDNA(−).

§$P < .05$ BT vs. non-BT.

Figure 1A-C shows values of TNF-α, IL-6, and NOx in MLNs in rats with cirrhosis and controls. In general, values were significantly higher in animals with BT when compared with controls and with rats with cirrhosis without BT (non-BT). When animals with negative-culture MLNs (non-BT) were divided in two subgroups, according to the presence or absence of bactDNA, similar findings were observed as described previously in serum and AF.

Values observed in the subgroup of animals receiving norfloxacin were similar to those in animals without BT (non-BT) and absence of bactDNA, and the statistical differences are detailed in Table 2 and Fig. 1A-C.

Discussion

We have demonstrated that the presence of bactDNA of a certain bacterial species in blood, AF, or pleural fluid in rats with experimental cirrhosis is always associated with its simultaneous presence in MLNs in either culture-positive or culture-negative samples. Furthermore, the presence of bactDNA in MLNs in this experimental model is associated with a marked local and systemic inflammatory reaction, indistinguishable between culture-positive or negative samples. This finding implies that the detection of bactDNA may be considered as a marker of BT in the setting of cirrhosis. Because the pathogenesis of BT and spontaneous bacterial peritonitis is similar in this experimental model of cirrhosis and ascites in rats and in patients with cirrhosis, we can assume that similar results could be observed in patients. Therefore, the presence of bactDNA in serum or AF in patients with cirrhosis could be considered a reliable indirect marker of BT. The fact that bactDNA has not been detected in any biological samples from animals receiving norfloxacin as selective intestinal decontamination, and that both cytokine and NOx levels are similar to those in controls, strongly supports this concept.

As shown in the current investigation, bactDNA may be present in culture-negative MLNs, and this might be attributable to the transfer of fragmented bacteria to MLNs carried by dendritic cells.[7] In our series, 7 of 19 animals showed BT (Table 1), a percentage similar to that of other previously published series.[1,12-14] In contrast, bactDNA was detected in 12 of 19 animals in MLNs, and the degree of concordance between culture-positive MLNs and the identification through nucleotide sequencing was 100%. Overall, bactDNA was detected in MLNs in seven cases without BT (culture-negative MLNs).

In our series, bactDNA was detected in blood and ascitic or pleural fluid in 11 of 19 animals, and culture-positive MLNs by the same bacteria species were observed in seven cases (63.6%). The remaining four cases showed the presence of bactDNA from the same bacteria species in culture-negative MLNs identified by means of PCR and nucleotide sequencing. The detection of bactDNA in biological fluids in a given animal was always associated with its simultaneous presence in MLNs as either culture-positive or -negative forms.

The presence of bactDNA in blood is associated with profound immune consequences. BactDNA contains repeated series of unmethylated CpG motifs, intracellularly recognized by toll-like receptor 9 after internalization in endosomic vesicles.[15] This complex formation induces the synthesis of different proinflammatory cytokines involved in the innate immune response[16] that have previously been reported in the cytoplasm[17] and in the

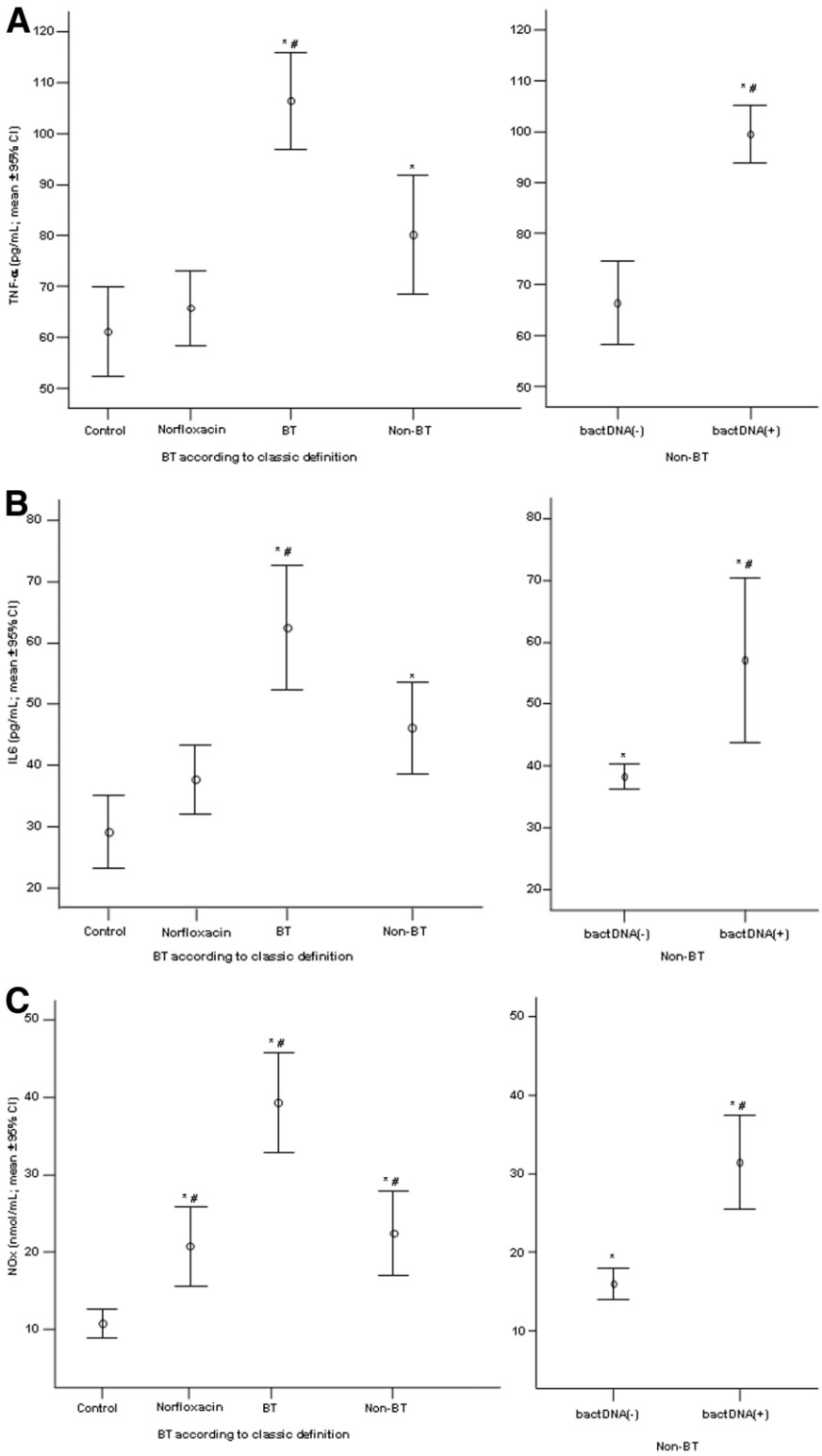

Fig. 1. (A) TNF-α measured in MLNs from rats with cirrhosis and controls. (B) IL-6 measured in MLNs from rats with cirrhosis and controls. (C) NOx measured in MLNs from rats with cirrhosis and controls. BT, bacterial translocation (culture-positive MLNs); non-BT, absence of bacterial translocation (culture-negative MLNs); non-BT bactDNA(−), culture-negative MLNs without the presence of bactDNA; non-BT bactDNA(+), culture-negative MLNs with the presence of bactDNA. *$P < .05$ compared with control; #$P < .05$ compared with bactDNA (−).

supernatant of cultures from patients with culture-negative, non-neutrocytic AF.[18] This is further associated with a higher NOx synthetic ability in cells obtained from patients with bactDNA.[18]

Dendritic cells are key elements in bacterial trafficking from the intestinal lumen to MLNs and are capable of sampling bacteria from the intestinal lumen through the emission of dendrites between adjacent epithelial cells,[19] carrying either living or dead bacteria to MLNs.[20] Then, bacteria may reach MLNs in different forms, and the result of culture of MLNs may be positive or negative. Once dendritic cells reach MLNs, both B and T cells are activated to induce an immune response, and the intensity of this response may vary according to the viability of the bacteria carried by dendritic cells.[7] In fact, the synthesis of TNF-α in culture-positive MLNs in patients with cirrhosis and presence of AF is increased,[21] and MLNs are the site of initiation of the immune response by enteric bacteria.[22]

As shown in Fig. 1, values of TNF-α, IL-6, and NOx measured in MLNs are similar between animals with classic BT (culture-positive MLNs) and those with culture-negative, bactDNA-positive MLNs. This likely means that the main factor inducing the inflammatory reaction is the presence of bacterial genome, and not the viability of the accessing bacteria, represented by the positivity of cultures. This further suggests that the concept of BT should be modified to include the presence of bactDNA in the sample.

In summary, detection of bactDNA in biological fluids in animals with experimental cirrhosis and ascites is associated with its simultaneous presence in MLNs, in either culture-positive or culture-negative MLNs. These data support our hypothesis that the presence of bactDNA in biological fluids in patients with advanced cirrhosis constitutes a marker for the diagnosis of BT. Moreover, because the presence of bactDNA in culture-negative MLNs is associated with an inflammatory reaction similar to that observed in culture-positive samples, we suggest that the concept of translocation might be expanded to consider BT as the presence of bacterial genome fragments in MLNs, irrespective of the result of microbiological culture.

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