

©2003, Acta Pharmacologica Sinica
Chinese Pharmacological Society
Shanghai Institute of Materia Medica
Chinese Academy of Sciences
<http://www.ChinaPhar.com>

Original Research

A striking correlation between lethal activity and apoptotic DNA fragmentation of liver in response of *D*-galactosamine-sensitized mice to a non-lethal amount of lipopolysaccharide¹

ZHOU Bing-Rong², Marina GUMENSCHHEIMER, Marina FREUDENBERG, Chris GALANOS³

Max-Planck-Institute for Immunobiology, Stübeweg 51, D-79108 Freiburg i Br, Germany

KEY WORDS lipopolysaccharides; *D*-galactosamine; apoptosis; DNA fragmentation

ABSTRACT

AIM: To observe whether challenge of bacterial lipopolysaccharide (LPS) with *D*-galactosamine (*D*-GalN) in mice will result in apoptotic characteristic of vital organs. **METHODS:** The experimental group of mice was challenged directly with bacterial LPS (0.05 µg) in the presence of *D*-GalN (20 mg), and the control group of mice was challenged either with bacterial LPS or with *D*-GalN alone. The organs including brain, kidney, heart, spleen, lung, and liver were removed at an indicated time point under ether anesthesia, and immediately homogenized, from which DNA was extracted. The DNA obtained from these organs was analyzed by agarose gel electrophoresis to determine whether the DNA laddering phenomenon existed. The amount of plasma LDH and GOT was detected in mice challenged with bacterial LPS in the presence of *D*-GalN, and either bacterial LPS or *D*-GalN alone. **RESULTS:** Apoptotic DNA fragmentation was initially seen at 4 h after challenge only in the livers of mice challenged with bacterial LPS and *D*-GalN, all mice in this group challenged with bacterial LPS and *D*-GalN died at 7 h after challenge; in contrast, the animals in the control group were all alive and the DNA was integral. **CONCLUSION:** The liver is the only specific target organ where apoptotic DNA fragmentation was present in mice treated with *D*-GalN and challenged with bacterial LPS and the liver impairment might be the critical cause of the lethality of mice elicited by bacterial LPS.

INTRODUCTION

Bacterial lipopolysaccharide (LPS) is a component located in Gram-negative bacteria wall. It usually leads to a high lethality due ultimately to the development of sepsis and shock. However, there is much significant discrepancy between various species of experimental animals after application of purified bacterial LPS upon these animals, even in the same animal species. Galanos^[1] reported that simultaneously administering the experimental animals with *D*-galactosamine (*D*-GalN) was able to tremendously increase the sensitivity of these ani-

¹ This work was supported by a fellowship to Dr ZHOU Bing-Rong from Max-Planck-Society, Germany, and partially supported by National Key Basic Research and Development Program (973 Program) No 2002CB513000.

² Now in *Department of Microbiology, Faculty of Basic Medicine, 2nd Military Medical University, Shanghai 200433, China.*
Phn 86-21-2507-0313. E-mail bingrong_zhou@hotmail.com

³ Correspondence to Dr Chris GALANOS.
Phn 49-761-510-8400. Fax 49-761-510-8403.
E-mail galanos@immunbio.mpg.de

Received 2001-11-29

Accepted 2002-08-03

mals to lethality of bacterial LPS, thereafter to extremely decrease the amount of bacterial LPS from 100 µg to 0.001 µg (ie, *D*-GalN is able to increase the sensitivity of mice to bacterial LPS up to 100 000-fold). The present study was designed to observe whether lethality of mice treated with *D*-GalN and challenged with non-lethal amount of bacterial LPS (0.05 µg) was due to concomitance of characteristic DNA fragmentation of apoptosis in some vital organs.

MATERIALS AND METHODS

Chemicals Bacterial LPS was prepared from *Salmonella abortus equi*^[2]. *D*-GalN hydrochloride, phenol, and chloroform were purchased from Carl Roth Company (Karlsruhe, Germany), and ethanol from JT Baker B V-Deventer Company (the Netherlands).

Mice Mice were handled, and cared for in accordance with the Guide for the Care and Use of Laboratory Animals in Germany, as legally required. The experimental protocol was carried out in compliance with Germany regulations and with local ethical committee guidelines for animal research. In this study, C57BL10/ScSn (ScSn), 10-week-old mice weighing approximately 22-25 g of both sexes were used and were housed in stainless steel wire cages with free access to food and water under specific pathogen-free conditions (SPF) of the Max-Planck Institute für Immunobiologie. There was no difference in either male or female of mice in this model. After about a one-week equilibration period, the animals were randomly divided into 6 groups for experiment.

Challenge of *D*-GalN-treated mice with bacterial LPS, lethality and induction of DNA fragmentation In this study, all reagents, including *D*-GalN and bacterial LPS were dissolved in PBS buffer (pH 7.2, Gibco BRL, Freiburg, Germany) with pyrogen-free. *D*-GalN (20 mg) and bacterial LPS (0.05 µg) were mixed into a total volume of 200 µL and injected via lateral tail vein in the experimental group; in the control group, the mice were administered with a total volume of either bacterial LPS (0.05 µg) or *D*-GalN (20 mg). In some experiments, the organs of interest were timely removed with time-course of lethality and emergence of characteristic apoptotic DNA fragmentation in mice challenged with bacterial LPS plus *D*-GalN, while in the other experiment, the organs of interest were removed 6 h after challenge with bacterial LPS in the presence of *D*-GalN. Blood was collected under ether

anesthesia by puncturing the axillary vessels; plasma thereof was prepared and stored at -80 °C until assay.

Removal of organs from mice and homogenization The organs of interest (liver, spleen, lung, heart, kidney, and brain) were removed under ether anesthesia, and placed in 1 mL of lysis buffer solution [Tris-HCl 10 mmol/L, pH 7.5, ethylene diaminetetraacetic acid 100 mmol/L, SDS 0.5 %, 0.1 mg/L proteinase K (Merck, Heidelberg, Germany), which was added shortly before use] and immediately pressed with the flat end of a sterile syringe piston through a metal mesh placed on ice. Alternatively, the organs were placed in a glass homogenizer containing 1 mL of lysis buffer, and homogenized at a high-speed vortexing on ice for 5 min. The suspensions were kept at -40 °C until DNA extraction.

Extraction and preparation of RNA-free DNA Usually 0.4 mL of the homogenized organ suspension described above was used for DNA extraction. The extraction procedure used was basically described as the literature^[3]. DNA from each sample was extracted twice with phenol, once with phenol-chloroform-isoamyl alcohol (PCI, 25:24:1; v:v:v) and once with chloroform. The procedure was detailed as follows: an equal volume of phenol (0.4 mL) was added to 0.4 mL suspension of homogenized organ, and mixed well by vortexing vigorously for 5 min. After centrifugation at 17 300×g at 4 °C for 10 min, the supernatant (aqueous phase) containing DNA was pipetted into a clean microcentrifuge tube. To this an equal volume of phenol was added and extraction repeated as described above. The resulting supernatant was extracted once with PCI and once with chloroform to remove residual phenol. After centrifugation at 17 300×g, 4 °C for 10 min, the supernatant was transferred to a clean tube and DNA precipitated by adding 2.5 volumes of absolute ethanol and sodium acetate to a final concentration of 0.3 mol/L and kept at -20 °C for 30 min (or overnight). Then it was centrifuged (17 300×g at 4 °C for 10 min) and pellet dissolved in TE buffer (Tris-HCL 10 mmol/L, pH 7.4, ethylene diaminetetraacetic acid 1 mmol/L, pH 8.0) containing RNase (0.1 mg/L) at 37 °C for 1 h. Subsequently, the suspension was re-extracted once with phenol, PCI and chloroform, and precipitated as described above. Finally, the concentration of DNA was measured in a spectrophotometer (PM6, Zeiss, Jena, Germany).

Analysis of DNA ladder by agarose gel electrophoresis Before electrophoresis, the DNA concentration of samples was adjusted to the approximately

equivalent concentration. Usually 2-3 μg of DNA sample was mixed with loading buffer (40 % sucrose, 0.25 % xylene cyanol, 0.25 % bromophenol blue) and placed in the well of agarose gel. The gel agarose (1.5 %, w/v) in electrophoresis buffer (Tris-botalic-ethylene diamine-tetraacetic acid buffer) was run at 100 volt until the purple tracer marker migrated to approximately 2 cm before the end of the gel. The gels stained with ethidium bromide (Sigma) at a concentration of 2 $\mu\text{g}/\text{L}$ were photographed under UV light using a Mitribishi Video Copy Processor (Gibco BRL, Heidelberg, Germany), and the graphs were stored in a magnetic disc. Alternatively the photos were printed out directly for immediate analysis of the results.

TNF α bioassay Plasma TNF α was measured in a cytotoxicity test using a TNF-sensitive L929 cell line in the presence of actinomycin *D* as described previously^[4]. The detection limit of the assay was 50 pg TNF per mL plasma.

Assay of lactate dehydrogenase (LDH) and glutamic-oxaloacetic transaminase (GOT) in mice plasma ScSn mice were challenged with 0.05 μg of bacterial LPS alone or with *D*-GalN (20 mg), and/or combination of both for 5 h, and exsanguinated under ether anesthesia. Plasma was collected and the amount of plasma LDH and GOT was detected by automated clinical chemistry analyzers (Roche/Hitachi 917, Mannheim, Germany) according to the instruction of the manufacturer in the central laboratory, Freiburg Klinik Medical Centre. Samples in triplicate were diluted with PBS (Gibco BRL, Heidelberg, Germany) before assay.

Statistical analysis Values were expressed as mean \pm SD and compared with Student's *t*-test. A *P* value of less than 0.05 was considered significant, and a *P* value of less than 0.01 as highly significant.

RESULTS

DNA fragmentation analysis In order to observe whether there was apoptotic phenomenon in some vital organs of the mice challenged with non-lethal amount of bacterial LPS in the presence of *D*-GalN, the animals in the experimental group ($n=6$ each group) were directly challenged with bacterial LPS (0.05 μg) plus *D*-GalN (20 mg). An obvious haemorrhagic lesion was seen in liver. When the samples containing DNA extracted from the selected organs of interest, including liver, spleen, lung, kidney, heart, and brain were run through agar-gel electrophoresis, the phenomenon

of DNA fragmentation was seen only in liver, not in other organs (spleen, lung, kidney, heart, and brain) of those mice challenged with a non-lethal amount of bacterial LPS in the presence of *D*-GalN (Fig 1A). On the contrary in the control group of mice challenged either with bacterial LPS (0.05 μg) or with *D*-GalN (20 mg) alone, DNA fragmentation was also absent in the organs of interest including spleen, lung, kidney, heart, and brain (Fig 1B). It was clearly indicated that liver was a vital target organ accompanying characteristically with apoptotic DNA fragmentation in mice.

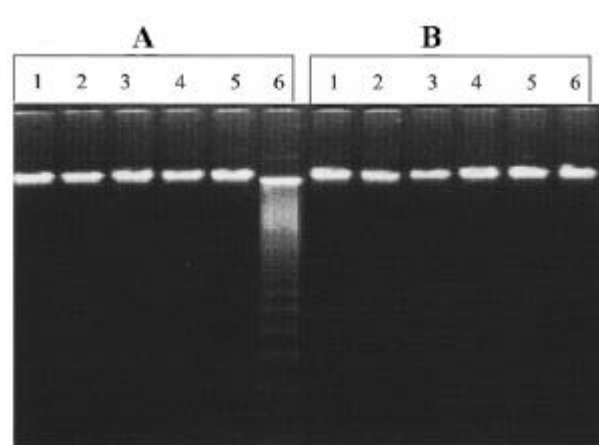


Fig 1. Organ specificity of LPS-induced DNA fragmentation in *D*-GalN-sensitized mice. Organs in part A and B from left to right were: brain (lane 1), kidney (lane 2), heart (lane 3), spleen (lane 4), lung (lane 5), and liver (lane 6). A: Challenged with LPS plus *D*-GalN; B: Challenged with *D*-GalN only.

Time-course in the onset of DNA fragmentation of *D*-GalN-treated mice challenged with bacterial LPS After challenge with bacterial LPS (0.05 μg) in the *D*-GalN (20 mg)-treated mice, the DNA fragmentation in liver of the affected mice was not seen at 3 h. Up to 4 h after challenge, a little part of DNA from liver of the affected mice was fragmented, and with the time forward, DNA in liver of the affected mice challenged with bacterial LPS in the presence of *D*-GalN was amputated off completely. At 5 h after injection of bacterial LPS and *D*-GalN, a typical DNA laddering phenomenon was seen in these mice (Fig 2).

Increased release of LDH and GOT in mice challenged with bacterial LPS/*D*-GalN A macroscopically hemorrhagic lesion of liver was accompanied with detectable DNA fragmentation in mice challenged with bacterial LPS/*D*-GalN. To evaluate whether it was simultaneously involved in the impact of plasma con-

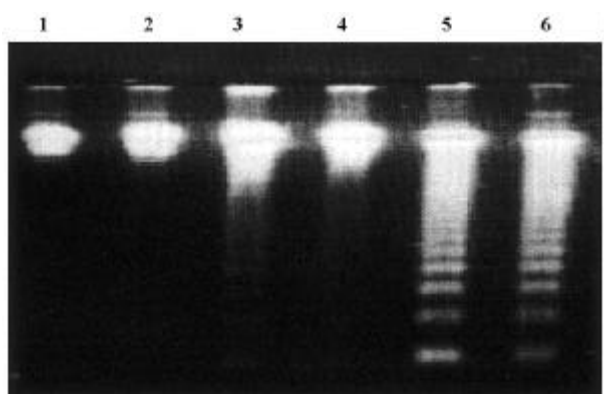


Fig 2. Kinetic experiment of DNA fragmentation in C57BL10/ScSn mice challenged with LPS plus *D*-GalN. C57BL10/ScSn mice were challenged with LPS (0.05 mg) plus *D*-GalN (20 mg) for different time. Livers were removed after sacrificing the mice under ether anesthesia at the indicated time point. About 3 mg of purified DNA in each lane was electrophoresed in agarose-gel. Lane 1, 2: 3 h; Lane 3, 4: 4 h; Lane 5, 6: 5 h after challenge, respectively.

tent of LDH and GOT, the activity of plasma LDH and GOT was determined in this study.

After challenge with bacterial LPS and *D*-GalN, plasma LDH was (1146±305) U/L and GOT was (256±72) U/L. However, in the control group, challenged with bacterial LPS (0.05 µg) alone, plasma LDH was (178±58) U/L, and GOT was (86±7) U/L, while in the control group challenged with *D*-GalN alone, plasma LDH was (278±58) U/L and GOT was (133±3) U/L. The difference of plasma LDH and GOT between the experimental and control groups was significantly different ($P < 0.01$, Fig 3). The results indicated that increased release of LDH and GOT from liver into plasma in mice challenged with bacterial LPS and *D*-GalN was intimately related to the onset of apoptotic reaction in liver.

Lethality of mice challenged with *D*-GalN and non-lethal amount of bacterial LPS There was no lethality (up to 24 h after challenge) in the control group challenged either with bacterial LPS or with *D*-GalN alone. However, all mice in the experimental group challenged with bacterial LPS and *D*-GalN died at 7 h after challenge. The results showed that lethality of mice was intimately correlated with hepatic lesion with onset of apoptotic DNA fragmentation (Tab 1).

DISCUSSION

LPS is an important pathogenic substance existing in the cell wall of Gram-negative bacteria. Injection

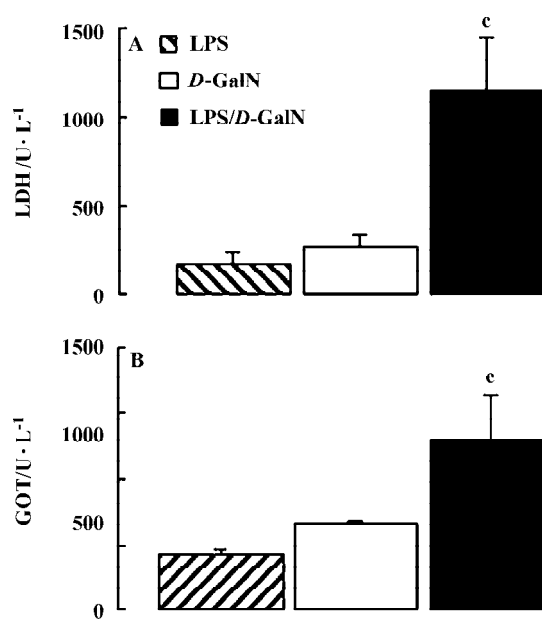


Fig 3. Increased release of LDH and GOT in mice treated with LPS plus *D*-GalN. Plasma level of LDH (A) or GOT (B) in ScSn mice 5 h after challenge with LPS (0.05 mg) in the presence of *D*-GalN, and with LPS or *D*-GalN alone were analyzed. $n = 6$. Mean±SD. * $P < 0.01$ vs LPS or *D*-GalN alone group.

Tab 1. A striking correlation between lethality of the mice challenged with non-lethal amounts of bacterial LPS plus *D*-GalN and DNA fragmentation in liver.

Challenge	Lethality (dead/total)	DNA fragmentation in liver
LPS/ <i>D</i> -GalN	6/6	+
LPS	0/6	-
<i>D</i> -GalN	0/6	-

+ fragmentation present; - fragmentation absent.

of purified bacterial LPS in experimental animals such as mice is capable of imitating the pathogenesis during the infection by Gram-negative bacteria. It is clear currently that LPS molecules do not directly induce the pathogenesis roles, but rather their effects are caused indirectly through the action of endogenous mediators, such as TNFα^[5,6] that is produced after interaction of bacterial LPS with specific targets in the organism. Yet, in this study, the activity of plasma TNFα was not detectable (data not shown) due to the low amount of bacterial LPS (0.05 µg only) applied. The reason may be that LPS was insufficient to induce these mice to

produce free forms of TNF α in circulation, though it was still able to produce local TNF molecules in liver, in particular by Kupffer cells, which was not determined in this study. Much rationally, when TNFR₂ deficient mice (TNFR₂^{-/-}) were challenged with the equivalent amounts of both LPS and *D*-GalN, as was applied in ScSn mice, both DNA fragmentation in liver and the lethality of mice were still present (data not shown), suggesting that lethality of mice and DNA fragmentation in liver were involved with TNF α molecules which were further signalled through TNF receptor 1.

It is known that biopharmacological effects of *D*-GalN are confined to hepatocytes, which is due to the lower level of uridine triphosphate (UTP) in liver^[7]. This decrement impairs biosynthesis of RNA and other macromolecular cell constituents (such as membrane glycoproteins and glycogen), resulting in damage and ultimate death of hepatocytes, and therefore greatly increasing the sensitivity of mice not only to bacterial LPS, but also to TNF α ^[1,8-10]. However whether the lower level of UTP in liver by *D*-GalN also causes damage to protective proteins in liver at either transcriptional site or translation level, is merely assumption so far. Nevertheless, in the case of mice challenged with bacterial LPS/*D*-GalN, these results in both DNA fragmentation in liver and lethality of the affected mice suggested that there was a close correlation between hemorrhage of the damaged liver and apoptosis in liver in the affected mice.

DNA fragmentation is a vital characteristic in apoptosis. With respect to the mechanism, it has been known that there are plenty of species of endogenous DNases existing as a status of zymogen in the cytoplasm. DNase molecules can recognize a specific sequence between adjacent chromosomes. When the zymogen of a given DNase is activated, DNA molecules within cells will be chopped up into various fragments with different lengths, thus leading to DNA fragments with 180-200 bp and their integral times, where a typical DNA laddering can be seen^[11-13] in agarose-gel after electrophoresis, which has been regarded as a vital marker in apoptosis that is distinctively different from necrosis. In this study, separate exposure of mice to a non-lethal amount of bacterial LPS or *D*-GalN was not able to induce DNA fragmentation in liver, DNA fragmentation can only be seen in the case of combination of both non-lethal amount of bacterial LPS and *D*-GalN. There is no clear evidence whether the effect of *D*-GalN can influence the activity of zymogen, such as turning

into activated DNase, or, increasing their activity following application of such lower amount of bacterial LPS. And further studies may be necessary. Generally, however, if there is an individual cell with demise via apoptosis, its content would not release, but rather it is engulfed by some adjacent cells such as macrophages, epithelial cells. Thus, after application of non-lethal amounts of bacterial LPS in the presence of *D*-GalN in this experiment, the releasable contents of LDH, GOT into circulation were significant higher than those in control group challenged with either bacterial LPS or *D*-GalN alone. The possible cause might be that injury to hepatocyte progress due to from initial apoptosis to secondary necrosis, ultimately destroying the integrity of cytoplasmic membrane in liver.

The lethality in concomitance with the characteristic marker of apoptotic DNA fragmentation in liver of mice treated by *D*-GalN and challenged with a non-lethal amount of bacterial LPS may be of clinical implication. Bacterial LPS usually abundantly existing in the gastrointestinal tract and permeating into the intestinal barrier^[14] and entering the circulation via the portal vein in some special situations, may become the cause of severe complications, such as multi-organ failure, in which the Kupffer cells may be activated by a minute amount of bacterial LPS and production of local TNF (free form and bound form of TNF molecules), leading to fulminating hepatic necrosis, disseminated intra-vascular coagulation (DIC), shock and even ultimate death^[15,16], especially in individuals who have been administered with some chemicals or infected simultaneously with virus which usually disturbs the synthesis of proteins including some proteins with vital function in host cells. Both cases are capable of affecting, and even increasing the reactivity of liver to bacterial LPS and/or TNF in given individuals. However actual mechanism of mortality in some individuals needs to be further elucidated, especially in those who are infected by Gram-negative bacteria and simultaneously by Gram-positive bacteria.

ACKNOWLEDGEMENTS We thank N GOOS and H STÜBIG for their excellent technical assistances.

REFERENCES

- 1 Galanos C, Freudenberg MA, Reutter W. Galactosamine-induced sensitization to the lethal effects of bacterial lipopolysaccharide. Proc Natl Acad Sci USA 1979; 76: 5939-

- 43.
- 2 Galanos C, Luderitz O, Westphal O. Preparation and properties of a standardized bacterial LPS from *Salmonella abortus equi* (Novo-Pyrexal). Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene-Erste Abteilung Originale- Reihe A: Medizinische Mikrobiologie und Parasitologie 1979; 243: 226-44.
 - 3 Fady C, Gardner A, Jacoby F, Briskin K, Tu Y, Schmid I, *et al*. A typical apoptotic cell death induced in L929 targets by exposure to tumor necrosis factor. J Interferon Cytokines Res 1995; 15: 71-80.
 - 4 Freudenberg MA, Gumenscheimer M, Jack R, Merlin T, Schütt C, Galanos C. A strict requirement for LBP in the TNF α hyper-response of Propionibacterium acnes-sensitized mice to lipopolysaccharide. J Endotoxin Res 1997; 4: 357-61.
 - 5 Galanos C, Freudenberg MA. Mechanisms of endotoxin shock and endotoxin hypersensitivity. Immunobiology 1993; 187: 346-56.
 - 6 Beutler B, Cerami A. The endogenous mediator of endotoxic shock. Clin Res 1987; 35: 192-97.
 - 7 Decker K, Keppler D. Galactosamine hepatitis: key role of the nucleotide deficiency period in the pathogenesis of cell injury and cell death. Rev Physiol Biochem Pharmacol 1974; (71): 77-106.
 - 8 Freudenberg MA, Galanos C. Induction of tolerance to lipopolysaccharide (LPS)-D-galactosamine lethality by pretreatment with LPS is mediated by macrophages. Infect Immun 1988; 56: 1352-57.
 - 9 Leist M, Gantner F, Bohlinger I, Germann PG, Tiegs G, Wendel A. Murine hepatocyte apoptosis induced *in vitro* and *in vivo* by TNF-alpha requires transcriptional arrest. J Immunol 1994; 153: 1778-88.
 - 10 Leist M, Gantner F, Naumann H, Bluethmann H, Vogt K, Brigelius-Flohe R, *et al*. Tumor necrosis factor-induced apoptosis during the poisoning of mice with hepatotoxins. Gastroenterology 1997; 112: 923-34.
 - 11 Granville DJ, Carthy CM, Hunt DW, McManus BM. Apoptosis: molecular aspects of cell death and disease. Lab Invest 1998; 78: 893-913.
 - 12 Nagata S. Apoptotic DNA fragmentation. Exp Cell Res 2000; 256: 12-8.
 - 13 Nicholson DW, Thornberry NA. Caspases: killer proteases. Trends Biochem Sci 1997; 22: 299-306.
 - 14 Houdijk AP, Meijer C, Cuesta MA, Meyer S, Van Leeuwen PA. Perioperative anti-lipopolysaccharide strategies. Scand J Gastroenterol 1997; 222: 93-7.
 - 15 Hack CE, Aarden LA, Thijs LG. Role of cytokines in sepsis. Adv Immunol 1997; 66: 101-95.
 - 16 Heumann D, Glauser MP, Calandra T. Molecular basis of host-pathogen interaction in septic shock. Curr Opin Microbiol 1998; 1: 49-55.