

Background: Exposure to microorganisms elicits the production of cytokines. These soluble factors enhance several innate immune functions and regulate the ensuing specific immune response aimed at limiting the spread of infection.

Aim: This study was undertaken to quantify the plasma levels of pro-inflammatory cytokines during the course of primary *Listeria monocytogenes* and *Campylobacter jejuni* infection. Using an *in vivo* infection the relationship between endogenous cytokines and the bacterial number in the liver of infected animals was examined.

Methods: C57BL/6 mice were infected by the intraperitoneal route. At different time points we determined the number of colony-forming units of bacteria in the liver of infected animals and paralleled these with the plasma levels of interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6) measured by enzyme immunoassays.

Results: *L. monocytogenes* infection lasted 10–11 days. IFN- γ production occurred in the early phase but was more pronounced after day 4, following the appearance of specific immunity. The duration of experimental campylobacteriosis was 15 days. Early IFN- γ production was not significant but a progressive rise of this cytokine in plasma was seen during the second week post infection. Mice produced measurable amounts of plasma TNF- α immediately after being given viable *L. monocytogenes*, peaking on day 2–3 when the greatest number of bacteria was present in the examined organs. During *C. jejuni* infection plasma TNF- α was produced in a similar manner, but the highest concentrations were found a few days later than in listeriosis, in correlation with the different course of campylobacteriosis. The quantity of IL-6 increased and decreased in concordance with clearance of *L. monocytogenes* and the clinical status of the animals. *C. jejuni* did not promote the induction of this cytokine. This is to some extent an unusual finding. With respect to the role of IL-6 in Th2 responses and antibody production, the appearance of this cytokine in campylobacteriosis was more expected.

Discussion: During systemic bacterial infection, a network of pro-inflammatory cytokines is activated and blood levels of these cytokines are elevated, albeit inconsistently, with large individual variations and depending on microbial characteristics and structure.

Key words: Cytokines, Mice, Infection, *Listeria monocytogenes*, *Campylobacter jejuni*

Plasma cytokine response in mice with bacterial infection

Maja Abram^{1,CA}, Darinka Vučković¹, Branka Wraber² and Miljenko Dorić¹

¹Department of Microbiology, Medical Faculty, University of Rijeka, Brce Branchetta 20, HR-51000 Rijeka, Croatia; ²Institute of Microbiology and Immunology, Medical Faculty, University of Ljubljana, Zaloska 4, SI-61000 Ljubljana, Slovenia

^{CA}Corresponding Author

Tel: (+385)51 651 172

Fax: (+385)51 651 177

Email: amaja@mamed.medri.hr

Introduction

Inflammation is mediated by a variety of soluble factors, including cytokines. Systemic bacterial infection, sepsis and multiple organ failure were found to involve a rapid onset and stable circulation of numerous cytokines,¹ but whether they can serve as markers for ongoing bacterial infection and prognosis remains controversial.^{2–6}

Listeria monocytogenes is a gram-positive, non-spore forming bacterium that is well equipped for intracellular parasitism. Thus, efficient sterilizing immunity to *L. monocytogenes* is essentially T-cell-mediated and independent of antibody.^{7,8} *Campylobacter jejuni* is a slim, gram-negative, curved or spiral shaped rod. The pathophysiology and defence mechanisms of the host to *C. jejuni* infection are still poorly understood, but some clinical and experimental

evidence suggests that the humoral immune response is of particular importance.⁹ However, *Campylobacter* is not a typical extracellular bacterium, because it has been shown that it can survive inside the phagocytes and that phagocytosis actually prolongs its viability.¹⁰

Campylobacter is one of the food-borne pathogens that has emerged in recent years, *Listeria* similarly.¹¹ The reasons cited for their growing incidence are complex and intertwined: the rise in meals prepared away from home, longer travel routes for raw food shipments and industrial, mechanized farming. Despite the fact that both *Listeria* and *Campylobacter* enter the host via the oral route, both of the bacteria are known for their ability to induce systemic infection. *Listeria* may cause meningoencephalitis, still birth, miscarriage or neonatal infection.¹² *Campylobacter* is responsible for pancreatitis, cholecystitis, cystitis, Guillain-Barre syndrome and even miscarriage.^{13,14} We have previously shown that intraperitoneal inoculation of *C. jejuni* in mice, in spite of no visible signs of illness, resulted in bacterial dissemination and tissue invasion.¹⁵ Mice intraperitoneally injected with *L. monocytogenes* exhibited some generalized symptoms (inactivity, ruffled fur) and more prominent gross and histologic abnormalities in the examined organs.

The present study was designed to evaluate whether plasma concentrations of interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α) and interleukin 6 (IL-6) were changed in C57BL/6 mice with primary listeriosis or campylobacteriosis and whether they correlate with bacterial clearance from the liver of infected animals.

Materials and methods

Mice

C57BL/6 (haplotype H-2b) mice of both sexes, aged ten weeks were obtained from the breeding colony at the Medical Faculty, University of Rijeka. They were kept in plastic cages and given standard laboratory food (Standard pellets, Faculty of Biotechnology, Domžale, Slovenia) and water *ad libitum*. The experiments were conducted according to the laws and principles found in the *International Guiding Principles of Biomedical Research Involving Animals* by the Council of International Organisations of Medical Science. The principles are also in accordance with the *Statute for Laboratory Animals of the Croatian Society for Laboratory Animals*.

Bacterial strains

Listeria monocytogenes haemolytic EGD strain (serovar 1/2a) was obtained from the Clinic of Infectious Diseases, Leiden, The Netherlands. The strain was kept virulent by *in vivo* passage. *Campylobacter*

jejuni was a clinical isolate from a patient with severe diarrhoea, obtained from the Department of Public Health, Rijeka. It was grown in a microaerophilic atmosphere (Generbox microaer, bioMérieux, Marcy-l'Etoile, France) at 42°C on blood agar plates supplemented with 5% sheep blood.

Route of infection

Mice were infected intraperitoneally with $0.5-1 \times 10^6$ *L. monocytogenes* or $0.5-1 \times 10^9$ *C. jejuni* in a total volume of 0.2 ml. The dose of viable bacteria was estimated at the time of infection based on the turbidity of the bacterial suspension and was subsequently measured by counting the number of colony-forming units (CFU) formed on blood agar plates after incubation.

Cytokine assay

At different time points after infection, experimental animals (five mice per group) were anaesthetized with sodium pentobarbital and bled from the retro-orbital plexus with ethylenediamine tetra-acetic acid (EDTA)-treated Pasteur pipettes. The tubes were centrifuged and the plasma was separated and stored at -20°C until assayed. Concentrations of TNF- α , IFN- γ and IL-6 were determined, using mouse cytokine ELISA kits purchased from Endogen Inc (Cambridge, MA, USA). Assays were performed according to the manufacturer's instructions, and the results expressed in pg/ml. The detection limit for IFN- γ and IL-6 was <15 pg/ml and for TNF- α it was <10 pg/ml. All tests were performed in duplicate. The results are presented as mean values \pm SE of the mean (SEM).

In vivo clearance study

Aseptically removed livers were dissected and homogenized in 5 ml of sterile phosphate-buffered saline. Serial ten-fold dilutions of the homogenates were plated and colonies were counted after 24 h incubation at 37°C for *Listeria* or 48 h incubation at 42°C in a microaerophilic atmosphere for *Campylobacter*. Bacterial titres are expressed as log₁₀ of cfu per liver.

Statistical analysis

The test for significance used in all cases was the Student's *t*-test.

Results

Bacterial clearance from the liver of infected C57BL/6 mice

Intraperitoneal injection of *L. monocytogenes* and *C. jejuni* led to different growth curves of the bacterium

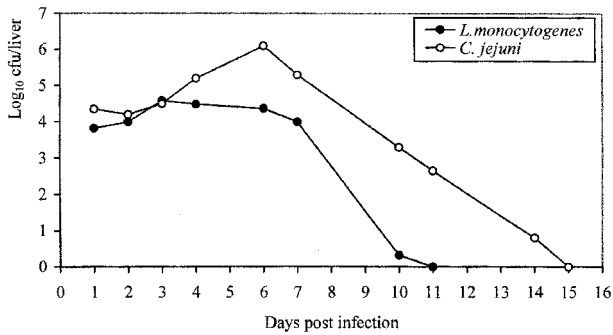


FIG. 1. Growth curve of *L. monocytogenes* and *C. jejuni* in the liver of C57BL/6 mice infected intraperitoneally with $0.5-1 \times 10^6$ viable *Listeria* and $0.5-1 \times 10^9$ *Campylobacter*. Each point represents the median value of bacteria recovered from groups of five mice. Data are expressed as \log_{10} cfu per liver.

in the mouse livers (Fig. 1). Listeriosis in the liver lasted for ten to eleven days. The bacteria were found in the liver from the first day after inoculation, but in a markedly lower number than inoculated. However, the number of *Listeria* increased and reached a peak 3 days post infection (p.i.). Eleven days p.i. sterile clearance of the bacteria from the liver was achieved. In the case of primary campylobacteriosis the bacteria were also found in the liver of the infected animals on the first day post intraperitoneal injection, but the highest number of bacteria was reached 6 days p.i. The infection in the liver was terminated 15 days after inoculation.

Plasma cytokine levels during primary listeriosis and campylobacteriosis

The levels of different cytokines (IFN- γ , TNF- α and IL-6) in the plasma of the animals infected with *C. jejuni* (Fig. 2A) or *L. monocytogenes* (Fig. 2B) were observed for ten days. IFN- γ was found in the plasma from the first day p.i. in mice infected with *Listeria* or *Campylobacter*. During the first four days the levels were similar in both infections, but did not differ much from that in the uninfected control animals (2382.6 ± 703.21 pg/ml). In the later phases of infection IFN- γ production was more pronounced. In campylobacteriosis the peak was reached on day 7 p.i. Interestingly, at this phase of infection, the concentration of this cytokine was much higher in mice infected with *C. jejuni* in comparison to those infected with *L. monocytogenes*.

On the first day after inoculation of *L. monocytogenes*, the mean level of plasma TNF- α was similar with the mean value found in control non-infected animals (55 ± 20.02 pg/ml), but, at the same time, it was significantly higher in comparison to campylobacteriosis ($P < 0.0001$). A significant increase ($P < 0.01$) and peak of TNF- α production was noticed on day 2 p.i., decreasing sharply thereafter. The second, lower peak, was observed on day 7, but did not reach

the basal values found in non-infected control animals. Finally, ten days p.i. TNF- α could not be detected in the plasmas of the *Listeria*-infected mice. During *C. jejuni* infection, plasma TNF- α was produced according to a similar biphasic pattern. The highest concentrations were found on day four after inoculation.

The dynamics of IL-6 production followed the number of *Listeria* in the livers of infected mice. After a strong increase at the beginning of the infection, the concentration of IL-6 further raised on day 2 ($P < 0.005$) and day 4 p.i., returning to the control value (110.6 ± 18.52 pg/ml) at the end of infection. On the contrary, *C. jejuni* did not induce an increased production of IL-6 during the entire course of infection.

Discussion

The role of cytokines in infection has been receiving increasing attention in recent years. To investigate to what extent the cytokine profiles are similar in different bacterial diseases, we used enzyme immunoassay to quantify the plasma levels of pro-inflammatory cytokines, IFN- γ , TNF- α and IL-6, in mice infected with two different bacteria: *Listeria monocytogenes* and *Campylobacter jejuni*.

L. monocytogenes is a gram-positive pathogen that evokes a strong T-cell-mediated immune response in infected animals.^{7,8} Once established within the cytoplasm, the bacteria multiply intracellularly and spread from cell to cell without being exposed to the extracellular host defences, such as complement or antibodies.¹⁶ On the other hand, gram-negative *C. jejuni* was considered to be only an extracellular parasite, suggesting that humoral immune responses are important in the control of *C. jejuni* infections. This is consistent with findings that *C. jejuni* isolates are generally susceptible to the bactericidal activity in normal human serum of both antibody and complement.^{17,18} However, recent data confirm its ability to invade some host cells especially mononuclear phagocytes and even enterocytes,^{10,19} but the possible role of cell-mediated immunity has to be further elucidated. In our study, although all infected mice were not displaying clinical signs of disease, we achieved a systemic infection after intraperitoneal injection of *Listeria* or *Campylobacter*. The infection was confirmed by following cfu-s in the liver of the injected animals. In spite of the dissemination, all the animals were capable of sterile elimination of bacteria. Bacterial clearance in the livers of infected mice depended on the bacterial species used. The difference between the two bacterial species was also in the time of onset and type of specific immune response to the microorganism. It is notable that once acquired immunity was established mice were very efficient at handling the infection.

The response of tissue to injury is characterized in the acute phase by increased blood flow and vascular

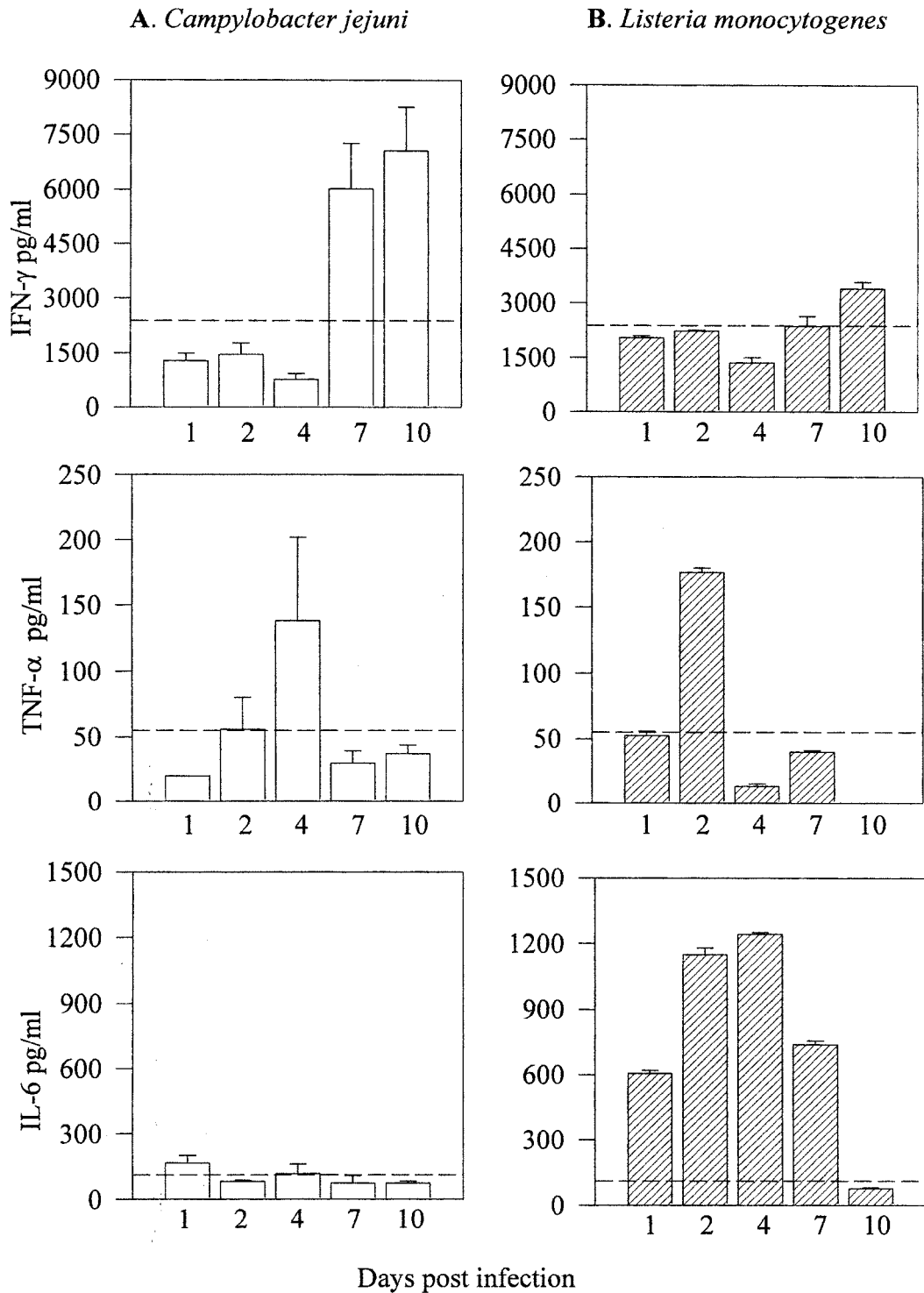


FIG. 2. The levels of IFN- γ , TNF- α and IL-6 in the plasma of C57BL/6 mice at different time points after intraperitoneal infection with $0.5-1 \times 10^9$ *C. jejuni* (A) or $0.5-1 \times 10^6$ viable *L. monocytogenes* (B). Data are expressed as mean values \pm SEM pg/ml. The basal values (dashed line) in non-infected, control mice were: IFN- γ = 2382.6 ± 703.21 pg/ml; TNF- α = 55 ± 20.02 pg/ml and IL-6 = 110.6 ± 18.52 pg/ml. Significance: IFN- γ (*C. jejuni*) day 7 vs. 4 ($P < 0.005$); TNF- α (*L. monocytogenes*) day 2 vs. 1 and 4, day 7 vs. 10 ($P < 0.001$); (*L. monocytogenes* vs. *C. jejuni*) day 1 ($P < 0.0001$); IL-6 (*L. monocytogenes*) day 2 vs. 1 ($P < 0.005$), day 7 vs. 10 ($P < 0.001$); (*L. monocytogenes* vs. *C. jejuni*) day 1 ($P < 0.001$), day 2, day 4, day 7 ($P < 0.0001$).

permeability along with the accumulation of inflammatory mediators such as cytokines. IFN- γ is known to enhance MHC class I and II expression on nucleated cells and to stimulate many effector functions of mononuclear phagocytes. Its primary function *in vivo*

appears to be the activation of macrophages to kill intracellular pathogens such as *Mycobacteria*,²⁰ *Leishmania*²¹ or *Listeria*,²²⁻²⁴ but there is no such evidence for *Campylobacter*. In our study the initial production of IFN- γ was similar both in listeriosis and

campylobacteriosis. The mean values were not higher than the pre-infection level till day 7 p.i., when *C. jejuni* more effectively triggered the release of this cytokine, possibly because of an additional release of IFN- γ by CD4⁺ T-helper cells. Whether specific microbial products of *Campylobacter* are the major factors inducing this additional IFN- γ production is difficult to say.

TNF- α and IL-6 are typical examples of multi-functional cytokines involved in regulation of the immune response, haematopoiesis and inflammation. Their functions are widely overlapping but each shows specific properties. The production of TNF- α during bacterial infections can be either beneficial or detrimental to the host. On the one hand, increased circulating levels of TNF- α demonstrated during overwhelming gram-negative bacterial infections or experimental endotoxaemia^{25,26} lead to septic shock. On the other hand, it has been reported that TNF- α is crucial in anti-listerial resistance,²² but it could not be detected in the circulation of mice during a sub-lethal *Listeria* infection.^{27,28} In our experiment, *L. monocytogenes*-infected mice showed TNF- α plasma levels higher than *C. jejuni*-infected animals, especially during the first two days p.i. During campylobacteriosis TNF- α peaked on day four, decreasing rapidly thereafter. TNF- α levels in plasma may not reflect the synthesis of this cytokine by cells. A variety of cells show high-affinity surface receptors for TNF and are able to trap it efficiently.²⁸ This suggests that detectable plasma TNF- α represents the excess of produced TNF.

The most pronounced difference in inducing cytokine production between the two pathogens was seen in the case of IL-6. While *C. jejuni* did not stimulate IL-6 production, the concentration of this cytokine in the plasma followed the load of *L. monocytogenes* in the liver of infected mice. Importance of IL-6 production in primary listeriosis and its correlation with the severity of infection has been also stressed by other authors.²⁹⁻³⁰ Nevertheless, its exact mode of action still remains to be explained. One possible mechanism, through neutrophil stimulation has been suggested,³⁰ but these molecules could equally help in the effective clearance of *Listeria* through acute phase protein production and by means of other mechanisms as well. Lack of IL-6 production during experimental murine campylobacteriosis was to some extent an unexpected finding since IL-6 acts as a growth factor for mature B cells and induces their final maturation into antibody-producing plasma cells. However, this obviously supports the data provided by some authors that *C. jejuni* is not a typical extracellular bacterium.^{10,31}

The presented data point to the complex mechanisms involved in the response to these two pathogens. We were unable to identify significant association between peripheral cytokine concentrations and

clinical outcomes in both listeriosis and campylobacteriosis. The different cytokine pattern is probably due to different characteristics in cell structure of the two bacterial species. However, studies in which levels of various cytokines have been measured during disease may be important for our understanding of their exact role and relative importance in the pathogenesis of gram-positive and gram-negative bacterial infections.

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