Rev. Inst. Med. Trop. Sao Paulo
53(2):107-112, March-April, 2011
doi: 10.1590/S0036-46652011000200009

TNF-α PRODUCTION AND APOPTOSIS IN HEPATOCYTES AFTER *Listeria monocytogenes* AND *Salmonella* TYPHIMURIUM INVASION

Sânia Alves dos SANTOS(1), Dahir Ramos de ANDRADE JÚNIOR(1) & Dahir Ramos de ANDRADE(2)

SUMMARY

Invasion of hepatocytes by *Listeria monocytogenes* (LM) and *Salmonella* Typhimurium (ST) can stimulate tumor necrosis factor alpha (TNF-α) release and induce apoptosis. In this study, we compared the behavior of hepatocytes invaded by three *L. monocytogenes* serotypes (LM-4a, LM-4b and LM-1/2a) and by ST to understand which bacterium is more effective in the infectious process. We quantified TNF-α release by ELISA, apoptosis rates by annexin V (early apoptosis) and TUNEL (late apoptosis) techniques. The cell morphology was studied too. TNF-α release rate was highest in ST-invaded hepatocytes. ST and LM-1/2a induced the highest apoptosis production rates evaluated by TUNEL. LM-4b produced the highest apoptosis rate measured by annexin. Invaded hepatocytes presented various morphological alterations. Overall, LM-4b and LM-1/2a proved to be the most efficient at cell invasion, although ST adapted faster to the environment and induced earlier hepatocyte TNF-α release.

KEYWORDS: *Listeria monocytogenes; Salmonella* Typhimurium; Hepatocytes; Apoptosis; Tumor necrosis factor-alpha.

INTRODUCTION

Listeria monocytogenes is a gram-positive coccobacillus present in soil and water, found in the gastrointestinal tract of healthy human adults, that typically produces β-hemolysis[13]. It is a foodborne pathogen that, in addition to being a known cause of spontaneous abortion, can cause meningitis, meningoencephalitis, septicemia, and gastroenteritis, all of which are more severe in immunocompromised individuals. During infection, *L. monocytogenes* can cross the intestinal, blood-brain or placental barrier[3]. Approximately 25% of all cases of invasive listeriosis occur in pregnant women[13].

Although there are 13 described serotypes of *L. monocytogenes*, the serotypes 1/2a, 1/2b and 4b account for 95% of all human infections. Thus, they were the focus of the current study. These serotypes have different pathogenic potentials and responses to environmental conditions[16].

Salmonella is a gram-negative, rod-shaped bacillus possessing peritrichous flagella. It is classified as facultative anaerobic and chemoorganotrophic bacteria. *Salmonella* is found in foods, in the environment, and in humans, as well as in other animals (warm-blooded and cold-blooded). It is pathogenic for humans and for many animal species, causing typhoid fever, enteric fevers, gastroenteritis, and septicemia[21].

Salmonella Typhimurium can access systemic tissue (mainly spleen and liver) via the lymphatic system and the Peyer's patches. In a second pathway, phagocytes are believed to carry intestinal bacteria directly into the bloodstream without passing through the Peyer's patches[24].

The cytokine tumor necrosis factor alpha (TNF-α) plays a role in a number of immunological and biological processes, including immunostimulation, resistance to infective agents, resistance to tumors[7,8], sleep regulation[9], and embryonic development[25]. However, circulating TNF-α can increase the fatality in parasitic, bacterial and viral infections. In addition, TNF-α can induce necrotic or apoptotic cell death[1]. Nevertheless, its major role appears to be as an important mediator in resistance against invading pathogens[6].

The hepatocyte is the major site of bacterial replication in the liver. The characteristics of bacterial invasion of hepatocytes, as well as the consequences of such invasion, have not been fully evaluated. In this study, we compared the behavior of hepatocytes invaded by *S.* Typhimurium with those invaded by three *L. monocytogenes* serotypes. We quantified TNF-α release and determined the rates of cell death by apoptosis to answer which bacterium is more effective in the infectious process, producing more cytokine and apoptotic death.

MATERIAL AND METHODS

Primary culture of rat hepatocytes: The present study was conducted in the Laboratory of Bacteriology (LIM-54) of the Hospital das Clinicas, School of Medicine, University of São Paulo (USP). The study design was approved by the Research Ethics Committee of the institution.

(1) Laboratory of Bacteriology (LIM 54), Department of Infectious Diseases, School of Medicine, University of Sao Paulo, Sao Paulo, SP, Brazil. Av. Dr. Enéas de Carvalho Aguiar 500, 1º andar, sala 107, 05403-000 Sao Paulo, SP, Brazil. Tel/Fax: +55 11 3061-7029.
(2) Published posthumously.
Correspondence to: Sânia Alves dos Santos, Av. Dr. Eneas Carvalho de Aguiar 500, 1º andar, sala 107, 05403-000 Sao Paulo, SP, Brasil. Tel/Fax: +55 11 3061-7029. E-mail: saniasan@usp.br

Newborn female Wistar rats were obtained from the animal facilities of the USP School of Medicine. We used 15 to 20 rats per assay. The animals were anesthetized with ether prior to the surgical procedures, which were performed using an aseptic technique under biological safety cabinet, as described previously[19]. The material was then transferred to culture flasks (Nunc, Roskilde, Denmark) covered with laminin (50 µg/mL; Sigma, St. Louis, MO, USA). On the first day of culture, we used Williams' E medium enriched with the following supplements: insulin, 10^{-8} M; glucagon (Sigma), 10^{-9} M; growth hormone (Sigma), 10 µU/mL; epidermal growth factor (Sigma), 10 ng/mL; ampicillin, 100 µg/mL; streptomycin (Sigma), 100 µg/mL; and amphotericin (Sigma), 5 µg/mL. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air[19].

Bacterial culture: The *L. monocytogenes* serotypes 4a (LM-4a, ATCC 19114; American Type Culture Collection, Manassas, VA, USA), 4b (LM-4b, ATCC 19115; American Type Culture Collection), and 1/2a (LM-1/2a; Probac, São Paulo, Brazil) were cultured in brain heart infusion (BHI) broth (Merck, Darmstadt, Germany), whereas *S.* Typhimurium (Probac) was cultured on blood agar (Merck), all cultures being incubated for 24 h at 37 °C[5]. Subsequently, one colony of each bacterium was seeded into 10 mL of BHI broth at 37 °C until the log phase of growth. Then the bacteria were diluted at 1:100 in 200 mL of BHI broth. These flasks were incubated at 37 °C. Once every hour, 1 mL samples were collected in polystyrene cuvettes for the determination of absorbance using a spectrophotometer (Shimadzu, Kyoto, Japan). This was continued for the initial nine hours of incubation, after which the culture entered the stationary phase.

Bacterial invasion of hepatocytes: The bacterial inoculum was washed three times with PBS (pH 7.2) and centrifuged at 700g for five min. The inoculum was then diluted in RPMI-1640 medium (Sigma) at 60:1, without antibiotics. The resulting solution was added to each culture flask (3 mL/flask), and the cultures were incubated at 37 °C for two hours to allow bacterial entry[5]. The bacterial concentrations used were proportional to the cells number, expressed as multiplicity of infection (MOI), as follows: MOI 0.5 (ratio, 5×10^5 bacteria:1×10^6 cells); MOI 5 (ratio, 5×10^6 bacteria:1×10^6 cells); MOI 50 (ratio, 5×10^7 bacteria:1×10^6 cells); MOI 500 (ratio, 5×10^8 bacteria:1×10^6 cells); MOI 5000 (ratio, 5×10^9 bacteria:1×10^6 cells); and MOI 50,000 (ratio, 5×10^{10} bacteria:1×10^6 cells)[19].

At six incubation time points (1, 2, 3, 4, 5 and 6 hours), the supernatants were removed and stored at -70 °C for subsequent TNF-α quantification[23]. The flasks were then washed with PBS, and 0.5 mL of Trypsin-EDTA (Cultilab, Campinas, Brazil) was added to each flask in order to detach the cells. This was followed by centrifugation (700g for five minutes) in Falcon flasks (Becton Dickinson, Franklin Lakes, NJ, USA) with RPMI-1640 medium and 10% fetal bovine serum.

Quantification of the number of invasive bacteria: In order to determine the number of invasive bacteria, the cells were incubated for 30 minutes in RPMI-1640 medium with 10 µg/mL of gentamicin to remove the extracellular (adherent) bacteria, after which the pellets were treated with 0.1% Triton X-100 and plated onto 24-well plates (Nalge Nunc, Rochester, NY, USA). The plates were incubated for 24 hours at 37 °C.

Quantification of TNF-α by ELISA: The quantification of TNF-α produced by hepatocyte culture supernatants was performed using ELISA for rat TNF (Biotrak ELISA; GE Healthcare, Piscataway, NJ, USA) in accordance with the manufacturer´s instructions.

Detection of apoptosis by TUNEL and annexin V techniques using flow cytometry: For detection of fragmented DNA, we used the Fragment End Labeling Kit (FragEL kit; Calbiochem, Oncogene Research Products, Cambridge, MA, USA), in accordance with the manufacturer´s instructions using flow cytometry.

For the detection of early apoptosis, we used a commercial kit (Annexin V-FITC kit; BD Pharmingen, San Diego, CA, USA), in accordance with the manufacturer´s instructions using flow cytometry.

Cell morphology by electron microscopy: Isolated cells were counted, pelleted, and washed twice in PBS. The resulting pellet was resuspended in 2.5% glutaraldehyde (0.1 M, pH 7.3; Sigma) for a minimum of two hours at 4 °C. Following fixation, the cells were washed three times in PBS to remove glutaraldehyde. The pellet was infiltrated with 2% osmium tetroxide (Sigma) in s-collidine (0.1 M, pH 7.4) for two hours at 4 °C. After four washes with PBS, 0.25-0.5% uranyl acetate was added, and the preparation was allowed to incubate overnight. After subsequent washes with PBS+saline-glucose, the pellet was dehydrated in a graded series of alcohol, with acetone, for five minutes. The pellets were then embedded in Epon Polybed 812-Araldite+Acetone (1:1; Polysciences, Warrington, PA, USA) and incubated at 37 °C for three hours. In the subsequent step, the solution was changed to pure Epon Polybed 812 (Polysciences), and the pellets were incubated at 65 °C for three days.

The preparation was then sectioned. Sections were placed on copper mesh grids (Sigma) and examined under a transmission electron microscope (JEOL 1010; JEOL, Tokyo, Japan).

Statistical analysis: The reactions were evaluated considering the variation of TNF-α production (released in the culture medium) in time. They were separated for bacteria concentration as well as by strains serotypes. The linear regression was applied obtaining linearity value (r^2) higher than 0.8. Comparisons were made using ANOVA with Dunnett's post test.

To analyze the TUNEL and annexin V data, we calculated the area under the curve for each experiment. The values were expressed in UA (units of area). Then we applied linear regression and ANOVA with Student-Newman-Keuls post test. Values of $p < 0.05$ were considered statistically significant.

The terms "release rate" and "production rate" describe the quantification of TNF-α effectively released by hepatocytes. The term "production rate" was also employed for quantification of apoptosis.

The comparisons were made by variancy analyses test with Dunnet post-test considering as differential element of contrast the control group reaction. The comparisons were made intragroup among several concentrations and intergroup, among several strains.

RESULTS

Hepatocyte culture: On the fifth day of primary culture (experimental assay), cell viability was 91.2%, with an average of 1.8×10^6 hepatocytes/

mL of medium. The immunohistochemical test was positive for the cytokeratins AE1 and AE3. The other elements that proved the hepatocyte selectivity in the cell culture by this method were published previously[19].

***Growth kinetics of L. monocytogenes and S.* Typhimurium:** The LM-4b and LM-1/2a serotypes presented similar kinetic profiles, with a lag phase of seven hours and a log phase of up to 13 hours, at which point the culture reached the stationary phase. Serotype LM-4a presented similar kinetics to that of serotypes LM-4b and LM-1/2a, with lower absorbance but similar lag and log phase times. As can be seen in Figure 1, *S.* Typhimurium presented a shorter lag phase (four hours).

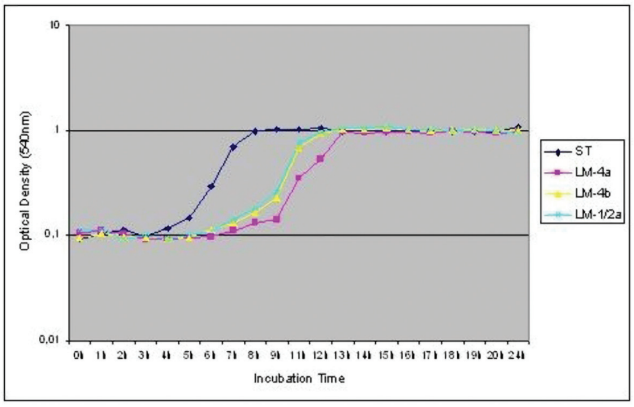

Fig. 1 - Growth kinetics of *S.* Typhimurium and of the LM-4a, LM-4b and LM-1/2a serotypes.

Recovery of bacteria from hepatocytes: We quantified the recovery of *S.* Typhimurium and *L. monocytogenes* from hepatocytes, at each of the six incubation time points (Tables 1 and 2). Recovery rates were highest for *S.* Typhimurium (approximately 10^5 times, 10^4 times, and 5×10^5 times higher, respectively, than for the LM-4a, LM-4b, and LM-1/2a serotypes). The recovery rates for the LM-1/2a and LM-4b serotypes were approximately 340 times and 300 times higher, respectively, than was that for the LM-4a serotype. There was a minimal difference between the LM-4b and LM-1/2a serotypes in terms of the recovery rate.

Analysis of TNF-α release by hepatocytes: The values of TNF-α released by hepatocytes at a fixed bacterial concentration (5×10^{10}) are shown in Table 3 (data analyzed by Dunnett's post test).

The rates of TNF-α release were highest for *S.* Typhimurium, followed by the LM-4a, LM-4b, and LM-1/2a serotypes in order.

Detection of apoptosis: Apoptosis was detected by TUNEL and annexin V technique in cultured hepatocytes, as shown in Tables 4 and 5.

Using the TUNEL technique, we found that *S.* Typhimurium and the LM-1/2a serotype were responsible for the highest apoptosis rates. These were followed by the LM-4b and LM-4a serotypes. Using the annexin V technique (analyzed by flow cytometry), we found that the highest apoptosis rate was achieved with the LM-4b serotype, followed by the LM-1/2a serotype and *S.* Typhimurium.

Microscopic analysis of infected cells: In order to evaluate the cytopathic effects of bacterial invasion, we used electron microscopy to observe hepatocytes after infection with *S.* Typhimurium or *L. monocytogenes*. After six hours of incubation, we observed hepatocytes with apoptotic morphology, characterized by atypical chromatin organization, with many vacuoles and apoptotic bodies (Fig. 2).

After three hours of incubation with the LM-4b serotype, hepatocytes presented apoptotic morphology—atypical chromatin organization, with many vacuoles and apoptotic bodies (Fig. 3).

Table 1
Recovery of *S.* Typhimurium from hepatocyte cultures

Incubation time	Percentage of recovered bacteria (%)			
	5×10^5	5×10^7	5×10^{10}	TOTAL
1 h	0	0	0	0
2 h	0	0	0	0
3 h	0	0	0	0
4 h	0	0	7.8×10^{-3}	7.8×10^{-3}
5 h	0	0.42	1.2×10^{-2}	4.32×10^{-1}
6 h	0.3	0.78	4.0×10^{-2}	1.12
TOTAL	0.3	1.2	5.9×10^{-2}	1.56

The labels 5×10^5, 5×10^7 and 5×10^{10} represent the bacterial concentration applied to hepatocytes.

Table 2
Recovery of LM-4a, LM-4b and LM-1/2a from hepatocyte cultures

Incubation Time	LM-4a (%)				LM-4b (%)				LM-1/2a (%)			
	5×10^5	5×10^7	5×10^{10}	TOTAL	5×10^5	5×10^7	5×10^{10}	TOTAL	5×10^5	5×10^7	5×10^{10}	TOTAL
1h	0	0	0	0	0	0	0	0	0	0	0	0
2 h	0	0	2.0×10^{-8}	2.0×10^{-8}	0	0	2.2×10^{-7}	2.2×10^{-7}	0	0	2.0×10^{-8}	2.0×10^{-8}
3 h	0	0	1.4×10^{-7}	1.4×10^{-7}	0	2.0×10^{-5}	4.2×10^{-7}	2.04×10^{-5}	0	2.0×10^{-5}	2.0×10^{-7}	2.02×10^{-5}
4 h	0	2.0×10^{-5}	2.0×10^{-7}	2.0×10^{-5}	0	1.2×10^{-4}	8.0×10^{-7}	1.21×10^{-4}	0	3.2×10^{-4}	3.2×10^{-7}	3.20×10^{-4}
5 h	0	8.0×10^{-5}	2.6×10^{-7}	8.03×10^{-5}	2.0×10^{-2}	3.6×10^{-4}	1.88×10^{-6}	2.04×10^{-2}	0	4.0×10^{-4}	5.8×10^{-7}	4.01×10^{-4}
6 h	2.0×10^{-3}	2.0×10^{-4}	4.0×10^{-7}	2.2×10^{-3}	4.0×10^{-2}	6.8×10^{-4}	3.0×10^{-6}	4.07×10^{-2}	4.0×10^{-2}	6.0×10^{-4}	8.0×10^{-7}	4.06×10^{-2}
TOTAL	2.0×10^{-3}	3.0×10^{-4}	1.0×10^{-6}	2.3×10^{-3}	6.0×10^{-2}	1.18×10^{-3}	6.32×10^{-6}	6.12×10^{-2}	4.0×10^{-2}	1.34×10^{-3}	1.92×10^{-6}	4.13×10^{-2}

The labels 5×10^5, 5×10^7 and 5×10^{10} represent the bacterial concentration applied to hepatocytes.

Table 3
TNF-α produced by hepatocytes invaded by *S.* Typhimurium, LM-4a, LM-4b and LM-1/2a

	TNF-α (pg/mL)				
	Control	*S.* Typhimurium	LM-4a	LM-4b	LM-1/2a
Production rate	1.257 ± 1.210	146.9 ± 18.38	94.71 ± 13.89	94.52 ± 15.66	58.16 ± 15.49

Data expressed as mean ± standard deviation.

Table 4
Apoptosis of hepatocytes invaded by *S.* Typhimurium, LM-4a, LM-4b and LM-1/2a—TUNEL technique

	TUNEL (%)				
	Control	*S.* Typhimurium	LM-4a	LM-4b	LM-1/2a
Apoptosis rate	11.57 ± 1.491	23.86 ± 1.614	15.91 ± 0.9343	21.14 ± 1.421	23.93 ± 1.263

Data expressed as mean ± standard deviation represent the percentage of cells in apoptosis.

Table 5
Apoptosis of hepatocytes invaded by *S.* Typhimurium, LM-4a, LM-4b and LM-1/2a—annexin V technique

	Annexin V (%)				
	Control	*S.* Typhimurium	LM-4a	LM-4b	LM-1/2a
Apoptosis rate	1.54 ± 1.40	12.51 ± 2.052	23.10 ± 3.481	26.61 ± 3.414	18.57 ± 2.497

Data expressed as mean ± standard deviation represent the percentage of cells in apoptosis.

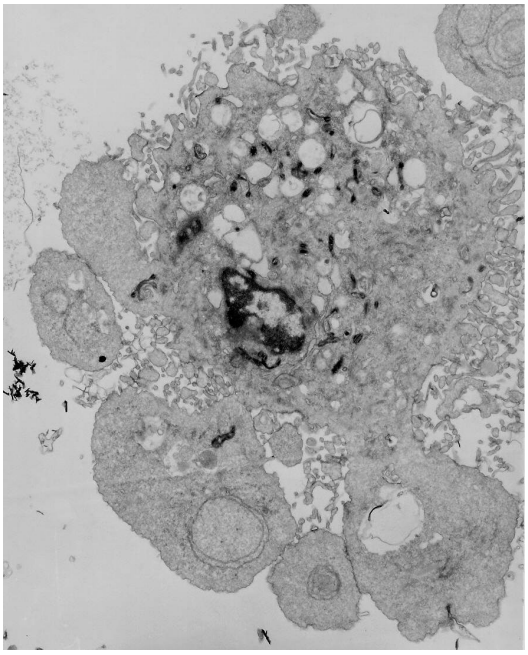

Fig. 2 - Photomicrograph of hepatocytes invaded by *S.* Typhimurium showing apoptotic bodies detaching from the cell (arrow a = apoptotic bodies; arrow b = chromatin arrangement; arrow c = vacuoles) (6 h, MOI 5000, ×5000)

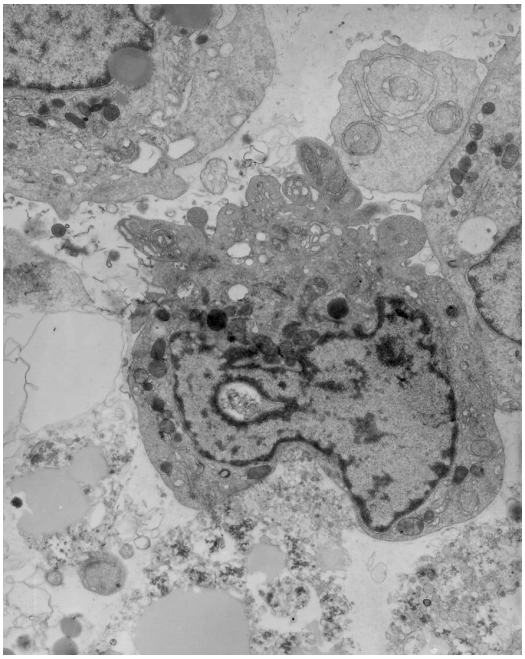

Fig. 3 - Photomicrograph of hepatocytes invaded by LM-4b showing apoptotic body formation (arrow a = apoptotic bodies; arrow b = chromatin arrangement; arrow c = vacuoles) (3 h, MOI 5000, ×5000)

DISCUSSION

Hepatocytes were chosen as the targets of *L. monocytogenes* and *S.* Typhimurium invasion because these bacteria preferentially invade hepatocytes rather than Kupffer cells in the liver[19,22-25].

By the study of growth kinetics of bacterial strains we found that LM 4b and LM 1/2a required a considerable period of *in vitro* adaptation prior to the onset of their multiplication. The peak absorbance values of LM 4b were higher than the other LM strains used, but the lag times and log phases were the same. We observed that *S.* Typhimurium also required a

shorter period of *in vitro* adaptation than *Listeria* spp. In the exponential growth phase, *S.* Typhimurium reached maximum absorbance after eight hours of incubation. Similar results have been obtained by other authors[14].

On the basis of our results, *Listeria* spp. adapted more slowly to environment conditions than *S.* Typhimurium, which required nearly half the time. Thus *Salmonella* spp. may be more efficient in their approach to the host. A longer lag phase increases the possibility that the host may develop an appropriate immune response.

To determine the invasiveness of *S.* Typhimurium and *L. monocytogenes,* we counted the numbers of bacteria recovered from hepatocytes. The number of *S.* Typhimurium recovered was higher than *L. monocytogenes* 4b and 1/2a. LM-4a was the least invasive bacterium by this criterion.

JARADAT & BHUNIA[8] showed that the LM-1/2a, LM-1/2b and LM-4b serotypes present great capacity for the invasion of Caco-2 epithelial cells. In this study the proportion of bacteria successfully invading the cells ranging from 0.14% to 0.32%. In addition, WOOD *et al.*[23] demonstrated the capacity of these bacteria to invade and multiply in murine hepatocytes.

In our study, *S.* Typhimurium (at MOI 50,000) was responsible for the highest quantity of TNF-α released in the culture medium by invaded hepatocytes. Using the kinetic growth curve we observed that *S.* Typhimurium adapted faster than others to this microenvironment. This phenomenon may explain the high rate of TNF-α released obtained. We speculate that ST approaches to the immune mechanisms occur more quickly than LM.

VÁZQUEZ-BOLAND *et al.*[22] showed that three of the 13 known *L. monocytogenes* serotypes—LM-1/2a, LM-1/2b, and LM-4b—alone account for more than 95% of all human and animal cases of listeriosis, although others, such as the LM-1/2c serotype, are often found as food contaminants[22].

SASHINAMI *et al.*[20] observed that TNF-α was produced continuously during persistent infection with *S.* Typhimurium, as well as the acute phase of salmonellosis. This phenomenon would promote the maintenance of attenuated bacterial growth in the host. This might partially explain the hepatocyte capacity for TNF-α production when invaded by bacteria. Another potential explanation for such production is the induction of apoptotic hepatocyte death, which would facilitate the immune response by the host immunity.

In the analysis of apoptosis evaluated by the TUNEL method, we observed that the rates of apoptosis for *S.* Typhimurium and the LM-1/2a serotype, although similar ($p > 0.05$), were higher than those obtained for the other bacteria studied.

Using the annexin V technique we found that the highest rate of apoptosis was obtained for LM 4b and the LM 4a. The annexin technique evaluates early apoptosis, when phosphatidylserine migrates from the internal to the external face of the plasmatic membrane, whereas the TUNEL technique evaluates apoptosis in the more advanced phase, when the cell presents cleavages in its chromatin, characteristic of this type of cell death.

The LM-4a serotype induced the lowest rates of TNF alpha release and apoptosis. The LM-4a serotype is rarely responsible for infections in human beings, and it shows limited ability to cause mouse mortality even via intraperitoneal inoculation[12]. These naturally avirulent strains contain a *PrfA* virulence gene cluster that is intact except for a defect in the *actA* gene. The LM-4a serotype also has a complete copy of the *internalin A* gene and a somewhat altered copy of the *internalin B* gene[10]. It is possible that our LM-4a serotype did not have the *PrfA* gene active which could explain its lower *in vitro* virulence and the greater delay in the apoptotic process. The LM-4a serotype has also been shown to induce a low rate of interferon γ production in murine macrophage cell line J774[11].

TNF-α can induce necrotic or apoptotic cell death[1]. The cell death caused by TNF-α may be more delayed in relation to that caused by other stimuli, such as the bacterium itself[4]. The LM-4b serotype induced TNF-α production by hepatocytes, as well as apoptotic death (by TUNEL and annexin V techniques). Because of these characteristics, the LM-4b serotype appeared to be the most efficient in the process of cell invasion (among the strains studied here). Although the LM-1/2a serotype was unable to induce significant production of TNF-α, it induced apoptosis, as assessed by TUNEL technique. This might indicate that TNF-α was not responsible for the apoptotic cell death in this case. The apoptosis would have occurred via other signaling pathways. The LM-1/2a serotype was also incapable of stimulating early apoptosis.

Salmonella-induced apoptosis could contribute to the escape of intracellular bacteria from the spent host cell following nutrient deprivation and termination of bacterial replication[7]. However, *S.* Typhimurium fails to induce apoptosis in epithelial cells. The induction of apoptosis in epithelial cells could be an attribute of the host response to bacterial invasion[7].

Listeria-infected hepatocytes produce chemoattractants for polymorphonuclear cells during early stages of the infection. Simultaneous production of several chemoattractants for polymorphonuclear cells by Listeria-infected apoptotic hepatocytes might promote infiltration of inflammatory phagocytes that control bacterial growth and the spread of the infection[7].

The morphologic analysis revealed differences among the studied bacteria in terms of their virulence. We observed that *S.* Typhimurium was able to induce significant morphologic alterations (rupture of the monolayer) at a lower concentration (MOI 500) than that required by the *Listeria* strains (data not shown). However, the *Listeria* strains invaded the hepatocytes in less time than did *S.* Typhimurium (one hour of incubation vs. three hours of incubation), although the *Listeria* strains required at least three hours to induce significant morphologic alterations. We can assume that *Listeria* spp. invade hepatocytes more rapidly than does *S.* Typhimurium, although this characteristic does not seem to represent a significant aggressive factor for the invaded cells.

It is known that the morphologic appearance of the apoptotic cell is of short duration, persisting for only a matter of minutes[17,18], and the apoptotic bodies in diverse forms is seen by only some hours before being phagocytosed[2,26]. Nevertheless, using electron microscopy, we were able to register apoptotic death induced by *S.* Typhimurium and *L. monocytogenes*.

Our findings add to the body of knowledge regarding the interaction between hepatocytes and two important invasive bacteria, *S.* Typhimurium and *L. monocytogenes*. This represents a first step toward treatment strategies for combating infections caused by these invasive bacteria. The tools of such strategies might be controlling the production of cytokines such as TNF-α, as well as regulating apoptotic cell death.

RESUMO

Produção de TNF-α e apoptose em hepatócitos após invasão por *Listeria monocytogenes* e *Salmonella* Typhimurium

A invasão de hepatócitos por *Listeria monocytogenes* (LM) e *Salmonella* Typhimurium (ST) pode estimular a liberação do Fator de Necrose Tumoral (TNF-α) e induzir a apoptose celular. Neste estudo comparamos o comportamento de hepatócitos invadidos por três sorotipos de *L. monocytogenes* (LM-4a, LM-4b e LM-1/2a) e por ST para entender qual bacteria é mais efetiva no processo infeccioso. Nós quantificamos a liberação de TNF-α pelos hepatócitos por ELISA e as taxas de apoptose pelas técnicas de anexina V (apoptose precoce) e TUNEL (apoptose tardia). A morfologia das células foi estudada também. A taxa de liberação de TNF-α foi mais alta em hepatócitos invadidos por ST. ST e LM-1/2a induziram as maiores taxas de apoptose pelo método TUNEL, enquanto LM-4b produziu as maiores taxas de apoptose por anexina V. Os hepatócitos invadidos apresentaram várias alterações morfológicas. Na análise do conjunto de dados, os sorotipos LM-4b e LM-1/2a provaram ser os mais eficientes na invasão celular, enquanto que ST adaptou-se mais rápido ao meio e induziu a liberação precoce de TNF-α pelos hepatócitos.

ACKNOWLEDGMENTS

This work was supported by the "Fundação de Amparo à Pesquisa do Estado de São Paulo" (FAPESP, Foundation for the Support of Research in the state of São Paulo).

REFERENCES

1. Beyaert R, Fiers W. Molecular mechanisms of tumor necrosis factor-induced cytotoxicity. What we do understand and what we do not. FEBS Lett. 1994;340:9-16.

2. Bursch W, Kleine L, Tenniswood M. The biochemistry of cell death by apoptosis. Biochem Cell Biol. 1990;68:1071-4.

3. Chaturongakul S, Raengpradub S, Wiedmann M, Boor KJ. Modulation of stress and virulence in *Listeria monocytogenes*. Trends Microbiol. 2008;16:388-96.

4. Cousens LP, Wing EJ. Innate defenses in the liver during *Listeria* infection. Immunol Rev. 2000;174:150-9.

5. Eckmann L, Kagnoff MF, Fierer J. Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. Infect Immun. 1993;61:4569-74.

6. Fiers W. Tumor necrosis factor. Characterization at the molecular, cellular and *in vivo* level. FEBS Lett. 1991;285:199-212.

7. Gao LY, Kwaik A. The modulation of host cell apoptosis by intracellular bacterial pathogens. Trends Microbiol. 2000;8:306-13.

8. Jaradat ZW, Bhunia AK. Adhesion, invasion and translocation characteristics of *Listeria monocytogenes* serotypes in Caco-2 cell and mouse models. Appl Environ Microbiol. 2003;69:3640-5.

9. Krueger JM, Fang J, Taishi P, Chen Z, Kushikata T, Gardi J. Sleep. A physiologic role for IL-1 beta and TNF-alpha. Ann N Y Acad Sci. 1998;856:148-59.

10. Liu D, Lawrence ML, Ainsworth AJ, Austin FW. Toward an improved laboratory definition of *Listeria monocytogenes* virulence. Int J Food Microbiol. 2007;118:101-15.

11. Liu D, Lawrence ML, Pinchuk LM, Ainsworth AJ, Austin FW. Characteristics of cell-mediated, anti-listerial immunity induced by a naturally avirulent *Listeria monocytogenes* serotype 4a strain HCC23. Arch Microbiol. 2007;188:251-6.

12. Liu D, Ainsworth AJ, Austin FW, Lawrence ML. Characterization of virulent and avirulent *Listeria monocytogenes* strains by PCR amplification of putative transcriptional regulator and *internalin* genes. J Med Microbiol. 2003;52:1065-70.

13. Lorber B. Listeriosis. Clin Infect Dis. 1997;24:1-11.

14. Malheiros PS, De Paula CM, Tondo EC. Cinética de crescimento de *Salmonella Enteritidis* envolvida em surtos alimentares no RS: uma comparação com linhagens de outros sorovares. Ciênc Tecnol Aliment. 2007;27:751-5.

15. Moura RAA, coord. Técnicas de Laboratório. 2ª ed. Rio de Janeiro: Atheneu, 1982.

16. Nelson KE, Fouts DE, Mongodin EF, Ravel J, DeBoy RT, Kolonay JF, *et al.* Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. Nucleic Acids Res. 2004;32:2386-95.

17. Russel SW, Rosenau W, Lee JC. Cytolysis induced by human lymphotoxin. Ann J Pathol. 1972;69:103-18.

18. Sanderson CJ. The mechanism of T cell mediated cytotoxicity. II. Morphological studies of cell death by time lapse microcinematography. Proc R Soc Lond B Biol Sci. 1976;192:241-55.

19. Santos SA, Andrade DR, Andrade Júnior DR. Rat hepatocyte invasion by *Listeria monocytogenes* and analysis of TNF-alpha role in apoptosis. Rev Inst Med Trop Sao Paulo. 2005;47:73-80.

20. Sashinami H, Yamamoto T, Nakane A. The cytokine balance in the maintenance of a persistent infection with *Salmonella enterica* serovar Typhimurium in mice. Cytokine. 2006;33:212-8.

21. Seeliger HP, Jones D. *Listeria.* In: Sneath PH *et al.*, editors. Bergey's manual of determinative Bacteriology. 9th ed. Baltimore: Williams and Wilkins; 1986. v. 2, p. 1235-45.

22. Vázquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Domínguez-Bernal G, Goebel W, *et al. Listeria* pathogenesis and molecular virulence determinants. Clin Microbiol Rev. 2001;14:584-640.

23. Wood S, Maroushek N, Czuprynski CJ. Multiplication of *Listeria monocytogenes* in a murine hepatocyte cell line. Infect Immun. 1993;61:3068-72.

24. Worley MJ, Nieman GS, Geddes K, Heffron F. *Salmonella* typhimurium disseminates within its host by manipulating the motility of infected cells. Proc Natl Acad Sci USA. 2006;103:17915-20.

25. Wride MA, Sanders EJ. Potential roles for tumour necrosis factor alpha during embryonic development. Anat Embryol (Berl). 1995;191:1-10.

26. Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature. 1980;284:555-6.

Received: 13 September 2010
Accepted: 20 January 2011