

Reactive nitrogen intermediates and monokines induce caspase-3 mediated macrophage apoptosis by anaerobically stressed *Salmonella typhi*

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Summary

A successful pathogen manipulates its host for its own benefit. After ingestion, on reaching the intestine *Salmonella* encounters the resident tissue macrophages. Rather than being destroyed by these professional phagocytes after internalization, *Salmonella* survives intracellularly. Invasive *Salmonella* has been reported to induce apoptosis of macrophages as a part of its infection process, which may allow it to avoid detection by the innate immune system. However, the induction of apoptosis under different host environments, including the anaerobic stress encountered by the pathogen in the gut, remains to be examined. The present study is aimed at investigating the apoptotic potential of *S. enterica* serovar Typhi (*S. typhi*) grown under anaerobic conditions simulating the *in vivo* situation encountered by the pathogen. Apoptotic cell death was determined by assessment of nucleosomal DNA and flow cytometric analysis. Evaluation of the data revealed that anaerobically grown *S. typhi* could induce apoptosis in significantly more number of macrophages compared to the bacterial cells grown under aerobic conditions. A significantly enhanced generation of reactive nitrogen intermediates and caspase-3 activity during macrophage apoptosis induced by anaerobic *S. typhi* correlated with the increased generation of tumour necrosis factor- α , interleukin (IL)-1 α and IL-6. The results indicate that reactive nitrogen intermediates and monokines induce caspase-3 mediated apoptosis of macrophages by *S. typhi* under anaerobic conditions. These findings may be relevant for clearer understanding of the *Salmonella*–macrophage interactions and may be of clinical importance in the development of preventive intervention against the infection.

Keywords: anaerobiosis, apoptosis, caspase-3, reactive nitrogen intermediates, *Salmonella typhi*, tumour necrosis factor

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Introduction

Salmonellae are Gram-negative bacteria, which cause a variety of symptoms ranging from a mild intestinal infection to life-threatening systemic infections. Salmonellae are facultative intracellular bacteria which do not require strict conditions for their growth. These are able to proliferate and survive under different environmental conditions, including food production and processing systems as well as a diverse array of stresses encountered inside the host.

During its life cycle, *Salmonella* encounters low oxygen conditions while invading intestinal epithelium, as well as in other tissues of the host during systemic infection. The invasive capabilities of *Salmonella* have been shown to be poten-

tiated in the presence of low oxygen, high osmolarity and alkaline pH, conditions that apparently signal the proper environment in the eukaryotic host [1]. Anaerobiosis has been reported to induce the invasiveness of *Salmonella* in cultured Madin-Darby canine kidney (MDCK) cells, human laryngeal tumour cells (HEp-2) and Henle cultured cell lines [2,3]. *Salmonella* under such conditions have also been shown to exhibit reduced LD₅₀, greater adherence and enhanced virulence compared to aerobic bacteria [4,5]. However, the effect of enhanced virulence of anaerobically grown *Salmonella* upon the fate of macrophages, which form the first line of host defence, is yet to be evaluated. *Salmonella* exploits macrophages as a crucial tool in the initiation of the disease. Rather than being destroyed by these professional

phagocytes, this pathogen is not only able to survive intracellularly, but also causes the apoptotic cell death of macrophages.

Apoptosis is an active cellular process of death triggered by certain stimuli. Apoptosis of host cells has been recognized to be involved in the regulation of immune response [6,7] and to occur during bacterial and viral infections [8]. Depending upon the pathogen, host cell apoptosis can be detrimental or beneficial to the pathogen. For example, the relevance of apoptosis by *Pseudomonas aeruginosa* has been demonstrated in favour of host defence [9]. *Salmonella*, however, has been reported to initiate apoptosis as a virulence strategy [10].

Recently, it has been observed that *S. typhi* grown under oxidative stress induces apoptosis in a significantly greater number of murine macrophages compared to the *S. typhi* grown under normal conditions [11]. However, during its infection route, anaerobic conditions are encountered by the pathogen long before the oxidative stress offered by the host within the macrophage milieu. Hence, it was worthwhile to investigate the potential of anaerobically stressed *S. typhi* to induce apoptosis of macrophages, which the pathogen may attempt in order to achieve immune avoidance.

Methods

Animals

Male inbred Balb/C mice, 4–6 weeks old (16–22 g in weight), used in the present study for extracting peritoneal macrophages, were procured from the Central Animal House, Panjab University, Chandigarh, India. The care and use of animals was in accordance with the guidelines of the institutional ethical committee.

Bacterial strain

The standard strain of *S. typhi* (Ty2), procured from Central Research Institute, Kasauli, India, was used in the present study. The strain was checked for purity and was characterized biochemically as well as serologically. The bacterial colony-forming units (CFU)/ml were calculated for infection of the macrophages.

Growth conditions

Bacteria were grown on nutrient agar plates and single colonies were used to prepare the seed culture. Inoculum 0.1% from seed culture was used to grow the bacteria under stress conditions. The bacteria were cultured by growing in nutrient broth under anaerobic conditions in accordance with the method of Schimann and Shope [12]. Anaerobic conditions were maintained by placing the culture in a Mackintosh anaerobic chamber in the presence of 85% nitrogen, 10% H₂ and 5% CO₂ at 37°C overnight.

Apoptotic cell death analysis

Isolation of peritoneal macrophages and their interaction with stressed bacteria. For isolation of macrophages, Balb/C mice were injected with thioglycollate broth (2.5 ml per mouse given intraperitoneally 4 days in advance) as described earlier [13] to achieve a better yield of macrophages. Macrophages were washed with RPMI-1640 (ICN Biomedicals, CostaMesa, CA, USA) at 4000 g for 10 min. Cell viability was checked with 0.2% trypan blue staining. For interaction, 10⁶ macrophages ml⁻¹ were infected with 10⁸ bacteria for 6 h in a humidified atmosphere containing 5% CO₂ at 37°C. A macrophage : bacteria ratio of 10⁶ : 10⁸ was chosen on the basis of multiplicity of infection (MOI). This MOI (1 : 100) was selected after assessing the macrophage viability as determined by trypan blue exclusion and macrophage cytotoxicity, by measuring the release of host cytoplasmic lactate dehydrogenase (LDH). At this MOI, the macrophage viability was found to be 56% after 6 h of interaction in comparison to the viability of the uninfected macrophages (> 85%) at the initiation of the experiment. LDH release by infected macrophages was found to be increased by 38% compared to the release by uninfected macrophages.

Assessment of nucleosomal DNA. DNA from cultured macrophages infected with the stressed *Salmonella* was isolated as described by Sambrook *et al.* [14] and nucleosomal DNA was assessed as described by Chanana *et al.* [11].

Fluorescein isothiocyanate (FITC)-labelled annexin V and propidium iodide staining. The flipping of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, which is a biochemical hallmark of apoptosis, is assessed by its binding with FITC-labelled annexin V (Roche Pharmaceuticals, Grenzach, Germany) for detection of apoptotic population [15]. After incubation time, cells were harvested using phosphate-buffered saline (PBS) + 0.02% ethylenediamine tetraacetic acid (EDTA), washed twice in PBS and suspended in 100 µl binding buffer (10 mM HEPES–NaOH, 140 mM NaCl, 2.5 mM calcium chloride, pH 7.4). Annexin V–FITC was added and cells were incubated for 15 min in dark conditions. After one wash, cells were resuspended in 200 µl binding buffer and counterstained with 1 µg/ml propidium iodide for determination of necrotic cells. Ten thousand cells per sample were analysed with a Becton Dickinson fluorescence activated cell sorter (FACSCalibur) equipped with a 15 mW, 488 nm air-cooled argon laser using CellQuest software.

Estimation of reactive nitrogen intermediates

Nitric oxide (NO) released from macrophages infected with stressed bacteria was measured by the determination of

nitrite and citrulline levels in the culture supernatants of the cells. Nitrite levels were measured by the method of Green *et al.* [16]. In this assay, nitrites in the sample react with a Griess reagent to form a purple azo dye. The supernatant was collected and an equal volume of Griess reagent was added. It was incubated for 10 min at room temperature and the optical density was recorded at 546 nm. Blank and standards (sodium nitrite; SRL, Mumbai, India) (0.1–1.0 μmol) were also run in parallel. The results were expressed as μmol of nitrite-formed/ 10^6 cells.

Citrulline levels were measured using the method of Boyde and Rahmattullah [17]. The supernatant (50 μl) was treated with 0.1 N HCl (450 μl) and 1.5 ml of reagent [prepared by mixing two parts of 550 ml distilled water + 250 ml of H_2SO_4 (95–98%) + 200 ml of O-phosphoric acid (85%) + 250 mg ferric chloride and 1 part of 0.5% w/v diacetyl monoxime + 0.01% w/v thiosemicarbazide]. Tubes containing the reaction mixture were immersed in a waterbath at 100°C for 6 min followed by cooling to room temperature. The optical density was measured at 530 nm. Blank and standard (citrulline; SRL) were run simultaneously. Results were expressed as μmol of citrulline-formed/ 10^6 cells.

Measurement of caspase-3 activity

Caspase-3 activity was measured using the colorimetric CaspACE assay system (Promega, Madison, WI, USA) following the manufacturer's instructions. After treatment with stressed bacteria, cells (1×10^7) were washed twice in ice-cold PBS and resuspended in cell lysis buffer (provided by the manufacturer) at a concentration of 10^8 cells/ml. The cells were lysed by four cycles of freezing and thawing and incubated on ice for 15 min. The cell lysates were centrifuged at 15 000 g for 20 min at 4°C and the supernatant fraction was collected and stored at –70°C. To measure caspase activity, the concentration of protein in each sample was determined [18]. One hundred μg of total protein (in a volume of up to 20 μl) from each sample was added to each well of a 96-well plate. Then 32 μl of caspase assay buffer (provided by the manufacturer), 2 μl dimethylsulphoxide (DMSO) and 10 μl of a 100 mM dithiothreitol (DTT) solution were added to each well. The final volume was adjusted to 98 and 2 μl of the caspase-3 substrate DEVD-P-nitroanilide (DEVD-pNA) was added to the reaction. The plate was incubated at 37°C overnight. Absorbance at 405 nm was determined with a spectrophotometric microplate reader (SpectraMAX plus; Molecular Devices, Sunnyvale, CA, USA).

Monokines assay

The cell-free culture supernatants were collected at the indicated time. The cytokines [interleukin (IL)-1 α , IL-6 and tumour necrosis factor (TNF)- α] activity in the culture supernatants was assessed by enzyme-linked immuno-

sorbent assay (ELISA) using commercially available mouse cytokine ELISA kits (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions. Briefly, 96-well microtitre plates precoated with monoclonal antibodies generated against mouse IL-1 α , IL-6 and TNF- α were used to capture the respective monokines produced in the culture supernatants of macrophages. The assays were visualized using streptavidin alkaline phosphatase conjugate for IL-1 α and TNF- α . For IL-6 the assay was visualized using a goat anti-rabbit-alkaline phosphatase conjugate and an ensuing chromagenic substrate reaction. The ELISA was sensitive to 0.2 pg/ml of the cytokines released. Linearity of the assay was assessed using different dilutions of the given standard. The precision profile was calculated by assaying replicates of standard as well as samples and then calculating the percentage coefficient of variation (%CV).

Statistical analysis

The statistical significance of results was determined by using the unpaired Student's *t*-test and two-way analysis of variance (ANOVA) with multiple comparisons. Data were considered significant at $P < 0.05$. The linearity and CV, particularly for monokines, were assessed as per the following formula: linearity = observed value/expected value \times 100; %CV = standard deviation/mean \times 100. Linearity in the range of 90–94% and %CV in the range of 5–10% was considered to be satisfactory, as these ranges indicate fairly good precision of the tests.

Results

Assessment of DNA ladder

The chromosomal DNA of macrophages infected with anaerobic stressed *S. typhi* was fragmented extensively into oligonucleosomes, clearly visible as a characteristic DNA ladder on agarose gels after 6 h (Fig. 1, lanes 5 and 6). However, this pattern was not detected at 4 h and 8 h post-infection of the macrophages. A single high molecular weight DNA band was observed in the control sample, in which the macrophages were not infected but the incubation time was 6 h (Fig. 1, lanes 1 and 2). Apoptosis is not in the biochemical repertoire of prokaryotes, but as a control *S. typhi* bacteria were subjected to phenol–chloroform extraction of DNA and no low molecular weight DNA was observed (Fig. 1, lane 3).

FITC-labelled annexin V and propidium iodide staining

Using FITC-labelled annexin V, the macrophage population treated with normal and anaerobic stressed *S. typhi* for 6 h showed 34% and 50% cells, respectively, as apoptotic (Fig. 2), in contrast to 3.0% apoptotic cells in the control

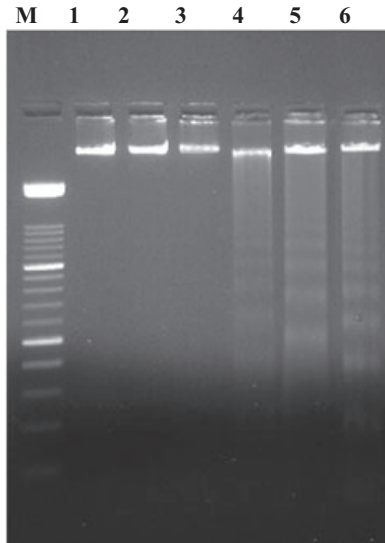


Fig. 1. Internucleosomal cleavage of macrophage DNA following *Salmonella* infection (multiplicity of infection 1 : 100) after 6 h of incubation; lane M: 100 base pairs (bp) DNA ladder, lanes 1 and 2: uninfected control, lane 3: DNA of *S. typhi*, lane 4: DNA of macrophages infected with normal *S. typhi*, lanes 5 and 6: DNA of macrophages infected with anaerobic *S. typhi*.

samples. The apoptotic cells were identified by flipping phosphatidylserine receptors on the outer leaflet of plasma membrane. Counterstaining with propidium iodide showed 6.0% and 7.11% as necrotic in macrophages treated with normal and anaerobically stressed *S. typhi*, respectively.

Estimation of reactive nitrogen species

Infection of macrophages with stressed *S. typhi* induced significantly increased ($P < 0.01$) nitrite and citrulline levels in the mouse peritoneal macrophages compared to the macrophages infected with *S. typhi* grown under normal conditions (Fig. 3a,b).

Caspase-3 activity

Caspase-3 activity was increased during apoptosis induced by anaerobic stressed *S. typhi* infection of macrophages (Fig. 4). Cells treated with these stressed bacteria showed a significant increase in caspase-3 activity compared to infection with normal *S. typhi* as well as to untreated control macrophages after 6 h treatment.

Monokine assays

Significantly enhanced levels of monokines IL-6, IL-1 α and TNF- α were observed when the macrophages were infected with anaerobically stressed *S. typhi* compared to the

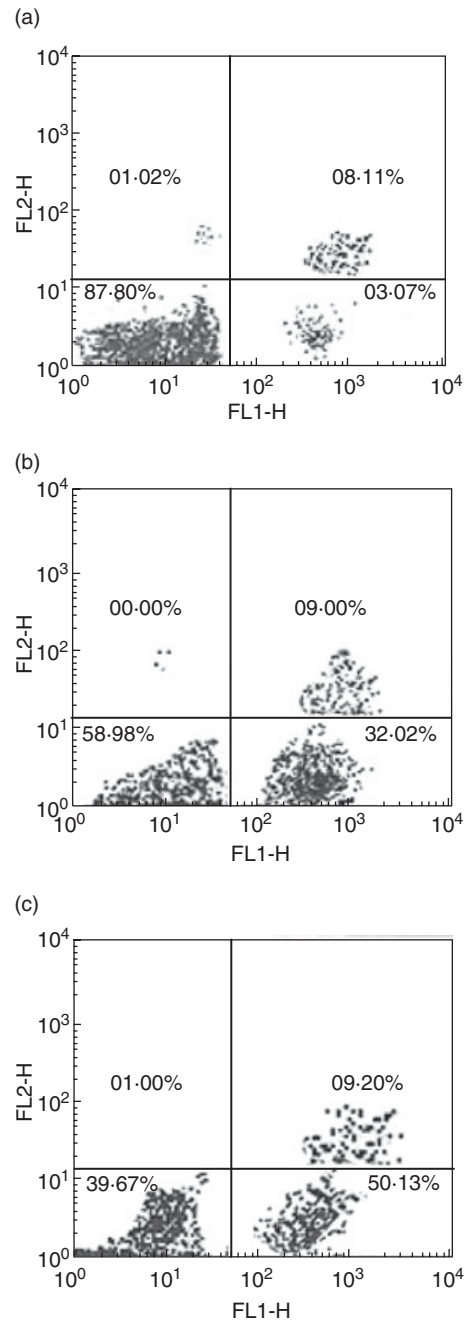


Fig. 2. Flow cytometric analysis showing annexin V binding properties of macrophages treated with *Salmonella typhi*. (a) Untreated cells shows only 3.07% of population as apoptotic (lower right chamber). (b) Normal *S. typhi* treated cells show 32% of population as apoptotic (lower right chamber). (c) Anaerobic *S. typhi*-treated cells show 50% of population as apoptotic (lower right chamber). Upper right chamber shows necrotic population which were stained with both propidium iodide and annexin-V.

untreated macrophages and the macrophages infected with *S. typhi* grown under normal conditions for 6 h (Fig. 5). Of the cytokines assayed, generation of TNF- α was found to be highest ($P < 0.001$).

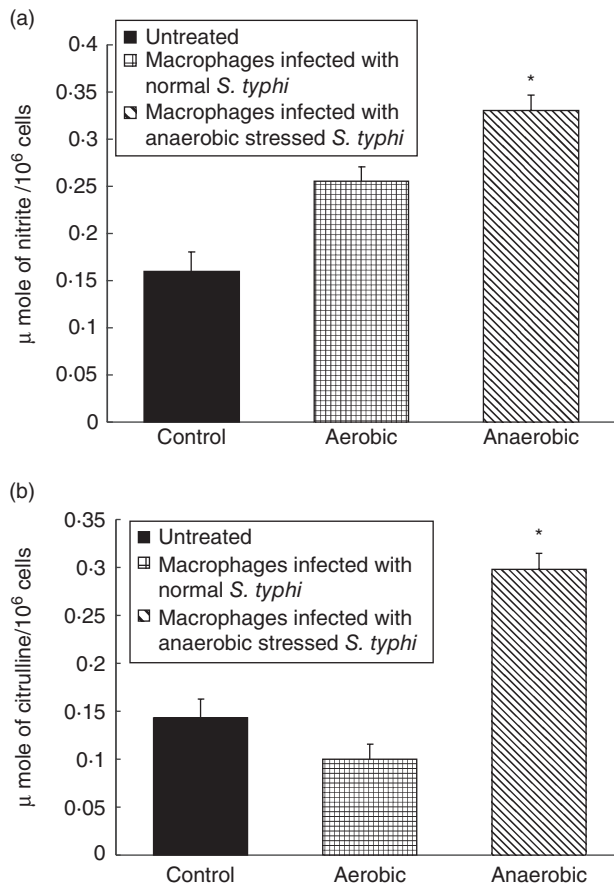


Fig. 3. (a,b) Levels of (a) nitrite and (b) citrulline ($\mu\text{mol}/10^6$ cells) in the culture supernatants of macrophages infected with *Salmonella typhi* grown under normal and stressed conditions. Values are expressed as mean \pm standard deviation of six different observations. *Significant difference ($P < 0.01$) from untreated control and normal *S. typhi*-infected macrophages.

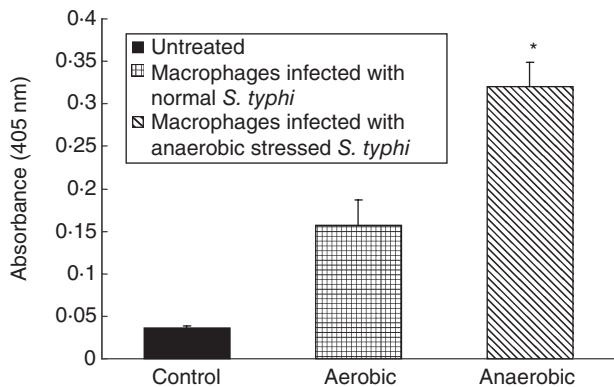


Fig. 4. Caspase-3 activity in the culture supernatants of macrophages infected with *Salmonella typhi* after 6 h incubation. Values are expressed as mean \pm standard deviation of six different observations. *Significant difference ($P < 0.01$) from untreated control and normal *S. typhi*-infected macrophages.

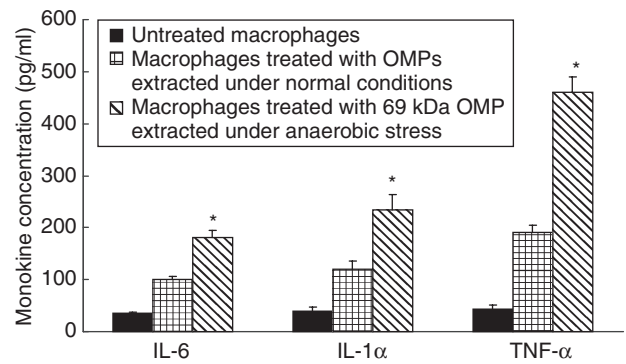


Fig. 5. Estimation of monokines levels (picogram/ml) in the culture supernatants of macrophages interacted with *Salmonella typhi* after 6 h of incubation. Values are expressed as mean \pm standard deviation of six different observations. *Significant difference ($P < 0.01$) from untreated control and normal *S. typhi*-infected macrophages.

Role of TNF- α in mediating bacteria-induced apoptosis

To evaluate the contribution of TNF- α in apoptosis, we determined whether TNF- α , produced as a part of the macrophage response to *Salmonella* infection, played a role in activating apoptosis after bacterial infection. Therefore, the apoptotic cells were again enumerated by acridine orange ethidium bromide co-staining after blocking the TNF- α activity using 20 $\mu\text{g}/\text{ml}$ of anti-TNF- α antibodies (Sigma, St. Louis, MO, USA) at the time when macrophages were infected with *S. typhi*, as described previously [11]. A significant reduction in the apoptotic cell population was observed (Fig. 6). As a control, normal goat IgG antibodies were added instead of anti-TNF- α antibodies.

Discussion

Macrophages serve essential functions as regulators of immunity and homeostasis [19,20]. As participants in native immunity, macrophages phagocytose, kill invading microorganisms and elaborate signalling molecules that amplify acute inflammatory responses. Macrophages also contribute to acquired immune responses by means of specialized functions that include antigen presentation and regulation of T cell responses. However, some bacterial pathogens, including *Salmonella*, have the capacity to induce macrophage apoptosis in order to elude innate immune responses and colonize the host successfully [21–24]. When a microbe such as *Salmonella* reaches the distal ileum, it must adapt to an anaerobic environment in order to carry out essential metabolic activities [2,25]. However, very little is known about the outcome of the microbe–macrophage interactions occurring under these situations.

In this study, it was observed that anaerobically stressed *S. typhi* cells were able to induce apoptosis of peritoneal macrophages after 6 h of interaction, as demonstrated by the results of assessment of nucleosomal DNA as well as

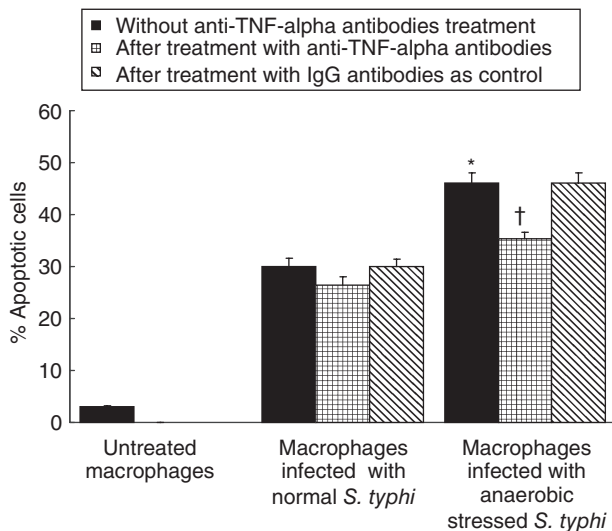


Fig. 6. Assessment of macrophage apoptosis (by acridine orange ethidium bromide co-staining) following infection with *Salmonella typhi* after 6 h of incubation after blocking tumour necrosis factor (TNF)- α activity. Values are expressed as mean \pm standard deviation of six different observations. *Significant difference ($P < 0.01$) from untreated control and normal *S. typhi*-infected macrophages. †Significant difference from anaerobic *S. typhi*-infected macrophages without anti-TNF- α antibody treatment.

flow cytometric analysis. The significantly increased number of macrophage apoptotic cells resulting from interaction with bacterial cells grown under anaerobic conditions may be due to the up-regulation or modification of certain genes and subsequently their corresponding proteins during the infection of macrophages with the pathogen under anaerobic conditions. It has been well documented that a number of genes including *orgA* (induced by anaerobiosis), *prgH* (a member of *PhoPQ* regulon) and invasion locus *hil* are expressed in *Salmonella* under anaerobic stress conditions [26]. It has been observed that bacteria growing under anaerobic conditions exhibit greater adherence as well as invasiveness and it has been proposed that increased invasiveness of anaerobically grown *Salmonella* was due to synthesis of at least two additional proteins [2]. It has also been reported recently that anaerobically grown *S. typhi* expresses 69 kDa OMP with enhanced intensity [27]. Expression of this protein under the anaerobic condition might be playing a role in induction of macrophage apoptosis.

NO has also been implicated in the induction of apoptosis of macrophages and monocytes. Reactive nitrogen intermediates (RNI) such as nitrites and citrulline are known to be the end-products of oxidative metabolism of labile nitric oxide and their quantification is regarded as an indicator of NO generation [28]. Significantly increased levels of nitrite and citrulline observed in the present study may contribute to *S. typhi* pathogenesis by inducing NO generation by the

macrophages. Increased levels of NO might have resulted in the increased activity of caspase-3 observed in this study, as NO has been reported to increase cellular ceramide level through the enhancement of caspase-3 activity [29]. The ceramide formation can induce several apoptotic signal pathways, including the release of mitochondrial cytochrome *c* into cytosol, the activation of caspase-9 and -3, the inhibition of protein kinase B/Akt and the suppression of Bcl-2 expression. Activation of caspase-3 has been reported to cleave the inhibitor for caspase activated DNase (ICAD), allowing CAD to enter the nucleus and degrade chromosomal DNA [30]. Increased activity of caspase-3 might be responsible for the nucleosomal fragmentation observed in the present study.

TNF- α , IL-1 α , IL-6 and IFN- γ have been reported to induce apoptosis in various cell types [31–33]. Evidence that TNF- α induces apoptosis via NO production is provided by the studies of Song *et al.* [34], in which it is demonstrated that TNF- α generation along with parallel increases in inducible type nitric oxide synthase (iNOS) mRNA expression and NO production induced apoptosis in wild-type mice cardiomyocytes. Schuerwegh *et al.* [31] have also suggested a significant role of NO in cytokine (TNF- α - and IL-1 α)-induced bovine chondrocytes apoptosis. Relationship of TNF- α with caspase-3 as well as NO [35] in activation of apoptosis has also been established. Accordingly, Gupta and Gollapudi [36] have also shown that increased apoptosis of T cells in aged humans is associated with increased activation of caspase-3 and TNF- α . In immune system-responsive cells, activation of caspase-3 has been shown to be required for apoptosis induced by Fas-FasL or TNF- α -TNF receptor interactions [37]. In the present study TNF- α -mediated apoptosis is also indicated with *S. typhi* grown under anaerobic conditions. These results are in concurrence with the earlier study wherein cellular sensitivity or resistance to TNF- α is correlated with decreased or increased levels of nitric oxide, respectively [34]. In our study, blocking of TNF- α activity in infected macrophage cultures using anti-TNF- α antibodies blocked, in part, the induction of apoptosis. These results are in accordance with an earlier study [38], wherein 20% reduction in epithelial cell apoptosis by *S. dublin* was observed.

Our results are suggestive of a central role of RNIs, caspase-3 and TNF- α in conjunction with IL-1 α and IL-6 in inducing the apoptotic cell death of macrophages following infection with *S. typhi* grown under anaerobic stress conditions. These findings may be relevant for the better understanding of the disease pathophysiology and for the future developments of diagnostic and preventive strategies.

Acknowledgements

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