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Mucin allows survival of *Salmonella typhi* within mouse peritoneal macrophages

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ABSTRACT

Salmonella typhi *is a facultative intracellular human specific pathogen. Both immunocompetent and immunodeficient mice are resistant to* ***S. typhi.*** *However, when they are infected with* ***S. typhi*** *suspended in mucin, the bacteria become pathogenic and infect peritoneal phagocytic cells. The* LD_{50} *for mice was* 10^5 *bacteria suspended in 5% mucin; mouse survival was approximately 48 hours after injection. A high number of bacteria was recovered from peritoneal cells; transmission electron microscopy disclosed a large number of vesicles filled with* ***S. typhi*** *cells in peritoneal cells from infected animals. The addition of mucin to cultures of the reticuloendothelial cell line J774.3 also allowed invasion of the mammalian cells with* ***S. typhi.*** *These data indicate that mucin allows intracellular survival of* ***S. typhi.***

INTRODUCTION

Salmonellae are responsible for a wide variety of infectious conditions in humans. Typhoid fever has been endemic in many countries like Chile, Perú, Mexico, Egypt, Indonesia and some Eastern European countries. In 1986, Edelman and Levine reported the occurrence of 1.25 x 10^7 new typhoid fever cases per year worldwide. Currently, neither the existing vaccines to prevent typhoid fever nor the vaccines in use for preventing Salmonellosis are effective (Germanier and Fürer, 1985; Isibasi, *et al.*, 1988; Cao *et al.*, 1992). The major difficulty in studying typhoid fever is that *S. typhi* is virulent only in humans, while laboratory rodents exhibit natural immunity.

S. typhi cannot invade and replicate in the mouse gut epithelium and reticuloendothelial cells. Macrophages from all mice strains efficiently engulf and kill *S. typhi* (Hormaeche, 1990). Nungester *et al.* (1936) described that mucin rendered mice susceptible to a variety of pathogenic and non pathogenic bacteria. Spaun (1964) used this approach to test the active protection of mice immunized with potential vaccines for preventing typhoid fever. More recently, Isibasi *et al.* (1992) and Chatfield *et al.* (1992) also used the inoculation of the pathogen suspended in mucin to determine protection against *S. typhi* in mice immunized with porins and *S. typhi* mutants, respectively. Mucin is the major component of the mucus that covers most of the gastrointestinal epithelial surface. A number of properties have been attributed to mucin, such us, cytoprotection, lubrication and prevention of the adherence of enteric pathogens (Marck and Sherman, 1991).

The value of the mucin model for studying typhoid fever was challenged by O'Brien (1982), who suggested that the death of the animals infected with large doses of *S. typhi* was due to the extracellular growth of bacte-

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ria and the high level of endotoxin rather than to intracellular growth.

The aim of this work was to gain further insight into the mechanism involved in the generation of septic shock that finally kills mice inoculated with *S. typhi* in mucin and to test whether the bacterial cells survive in the intracellular or extracellular compartment. To this end, we rescued bacteria from the intraperitoneal cavity at different times after infection; the intracellular or extracellular status of the microorganisms was determined by lysing the peritoneal exudate cells and plating them on agar. The intracellular location of the bacterial cells was investigated by transmission electron microscopy (TEM) on specimens taken at different times post-infection.

MATERIAL AND METHODS

Mice

Eight to 12 weeks old females or males inbred Balb/c mice (Ity^s genotype) were used. They were provided by the central animal house facility of the Pontificia Universidad Católica de Chile. Immunodeficient mice Balb/c scid/scid, supplied by Jackson Laboratories, Bar Harbor, Michigan, USA, were bred and maintained in our laboratory in isolation cages (scid, severe combined immunodeficiency).

Bacterial strains and LD_{50} determination

S. typhi Ty2, *S. typhimurium* LT2 and *E. coli* VCS 257 strains were kindly supplied by Dr. A. Yudelevich from the Pontificia Universidad Católica de Chile. The cells were picked from single colonies and grown in Luria broth at 37°C during 4 h with agitation. The cells were harvested when absorbance at 660 nm reached 0.3 OD (about 10^9 viable bacteria/ml). Crude mucin type II from porcine stomach was obtained from Sigma Chemical Co. and used at 5% final concentration. The number of viable bacteria in the culture was determined from ten fold serial dilutions in sterile PBS (phosphate buffer saline) and plating on Luria agar or McConkey agar plates.

Groups of 3 mice were infected intraperitoneally (i.p.) with different doses of bacteria suspended in 0.2 ml PBS or 0.2 ml mucin at 5% in PBS. Deaths were scored daily and the LD_{50} was determined by the method of Reed and Muench (1938). *E. coli* VCS 257 was used as non-invasive control.

*Effect of the interval elapsing between the injection of mucin and the subsequent inoculation of **S. typhi***

To determine the duration of the mucin effect on *S. typhi* virulence in mice, groups of 3 Balb/c mice were injected i.p. at different times with 100 µl mucin at 5%. Then all groups were inoculated at the same time with 4.0×10^6 bacteria. Deaths were scored for 48 h.

Preparation of peritoneal exudate cells (PEC) and plating of intracellular bacteria

Groups of 3 mice were inoculated with a lethal dose of *S. typhi* (1.24×10^6) suspended in 5% mucin or PBS. Mice were killed by cervical dislocation at different times (0, 2, 4, 18 and 22 h) and cells were recovered immediately by injecting 4 ml of sterile PBS into the peritoneal cavity. Peritoneal exudate was drawn through a small incision with the aid of a pasteur pipet and transferred into a 15 ml centrifuge tube. Viable bacteria present in the exudate at different times after infection were counted by plating on Luria or MacConkey agar.

To determine the presence of viable bacterial cells associated with or inside the cells, PEC was centrifuged at 1,000 rpm; the pellet was washed 3 or 4 times with sterile PBS and resuspended in 0.2 M acetic acid pH 2.5, for 6 min at 4°C (Haigler *et al.* 1980) to release loosely bound bacteria and then centrifuged. Dilutions of the supernatants were plated on MacConkey agar to count the bacteria cleared from the cell surface. Intracellular bacteria were released from the pellet after neutralization with 0.2 M sodium acetate pH 7.5, lysing the cells with 0.5 ml of a 0.1% Triton X-100 solution. Bacterial count was performed on Mac Conkey agar plates.

Preparation of peritoneal exudate cells for transmission electron microscopy (TEM)

PEC pellets were obtained from control (PBS- or mucin-injected) and infected animals according to the protocol described above but without acid wash. Pellets were fixed in 2% glutaraldehyde, 0.1 M sodium cacodylate buffer and post fixed in OsO_4 in 0.1 M cacodylate buffer. Pellets were dehydrated in acetone and embedded in EPON. Thin sections were double-stained with uranyl acetate and lead citrate, then examined under a Philips EM 300 transmission electron microscope.

*Culture of the J774.3 cell line and in vitro invasion of the microcultures by **S. typhi***

The cell line J774.3 is a subclone of the J774.2 line described by Bloom *et al.* (1978). The original cell line was obtained by Ralph *et al.* (1975) from a Balb/c reticulum sarcoma. The cells were cultured for 18 h on 24 or 96-well micro culture plates in Dulbecco's Eagle modified medium, supplemented with 10% fetal bovine serum without antibiotics for 18 h. Before infecting the cells, *S. typhi* was grown in Luria broth up to 0.2 OD. Approximately 10^5 J774.3 cells were co-cultured with 10^8 bacterial cells at different times, to allow infection in the presence of 2% mucin, or absence of mucin. After co-incubation, the cultures were washed with PBS and incubated for 3 h with culture medium supplemented with gentamicin, to avoid extracellular growth. Finally the cells were lysed with 0.5% deoxycholate for 20 min. The viable bacterial cells recovered from the micro-cultures were counted on Luria agar plates. The cultures were monitored by phase contrast microscopy. To observe the infected cultures on the light microscope, the infected and control J774.3 cultures were grown on glass coverslips and stained by Giemsa.

RESULTS

Lethal dose 50 determination

S. typhi suspended in PBS at doses up to 10^9 bacteria, injected i.p. was not virulent for Balb/c mice or Balb/c scid/scid. But, when suspended in 5% mucin and injected i.p., the bacteria were virulent and LD_{50} was 10^5 cells for both strains of mice. Mice survival post-infection was about 48 h. The non-invasive bacteria *E. coli* VCS were not virulent in mice when injected in mucin up to dosages of 10^9.

*Time course of mucin effect on **S. typhi** virulence in mice*

When mucin was injected i.p. before inoculating a lethal dose of *S. typhi*, its virulence enhancing effect lasted for about 2 h (Table I).

*Intracellular survival of **S. typhi** in mice intraperitoneally primed with mucin*

To determine whether mucin allowed survival of *S. typhi* in peritoneal phagocytic cells, doses of about 10^6 *S. typhi* resuspended in 5% mucin were used. At the onset of infection, recovery of viable extracellular bacterial cells was 10^5; it dropped to 10^4 at 2 h and increased to 10^6 after 22 h post-infection. In the intracellular compartment, a dramatic increase in the number of viable bacteria was observed 4 h post-infection; a further increase occurred up to 35 h post-inoculation (Fig 1). In contrast, in mice

TABLE I

Survival of mice inoculated with bacteria at different times after intraperitoneal injection of mucin

Time elapsed	Nº of mice dead	Nº of mice treated
0 min	3	3
30 min	3	3
1 hour	3	3
2 hours	3	3
4 hours	0	3
8 hours	0	3

Infective dose, 4 x 10^7 *S. typhi*. Deaths occurred ca 24 h after inoculation.

inoculated with *S. typhi* in PBS, few bacteria were recovered and an accelerated clearance of bacteria from the extracellular compartment was observed (data not shown).

*Transmission electron microscopy observation of peritoneal exudate cells from mice infected with **S. typhi** in mucin*

To determine the subcellular location and integrity of intracellular bacteria during infection with *S. typhi* in mucin, ultra-thin sections of PEC, taken at different times after infection, were observed with TEM. At time 0, intracellular bacteria were not observed in mice injected with *S. typhi* in mucin or in PBS. However, cells from mice injected with mucin alone presented a blistered cytoplasm (Fig 2). Four hours after inoculation, macrophages and polymorphonuclear cells from mice treated with bacteria in mucin, showed phagolysosome vesicles with bacterial cells at different stages of degradation, and some well preserved bacterial cells (Fig 3 A). In contrast, PEC from mice infected with *S. typhi* in PBS showed only bacterial debris (Fig 3 B). A similar picture was seen after 15 h post-infection, although there was a dramatic increase in the number of intracellular intact bacteria in mice injected with bacteria in mucin (Fig 4 A). The presence of copious lipid droplets and products of intracellular digestion in PEC from mice treated with *S. typhi* in saline buffer at 15 h post-infection, suggested an active antibacterial activity in the phagocytic cells (Fig 4 B). Close to the onset of the septic shock (22 h post-infection) a further increase in the number of intact bacteria was observed in phagocytic vacuoles. At this time, PEC cells from mice injected with bacteria in mucin showed evidence of cytopathic damage (Fig 5 A and 5 B).

*In vitro infection of the cell line J 774.3 with **S. typhi** in the presence of mucin.*

Figure 6 summarizes the effect of co-incubation time on the recovery of viable bacterial cells from microcultures; maximum recovery occurred at 2 h of co-incubation with mucin. Contrariwise, few bacteria were recovered from cultures without mucin. Giemsa staining of coverslips with J774.3 cell infected for 2 h with *S. typhi* in the presence of mucin, showed a large number of bacteria associated with the membrane of mammalian cells (Fig 7).

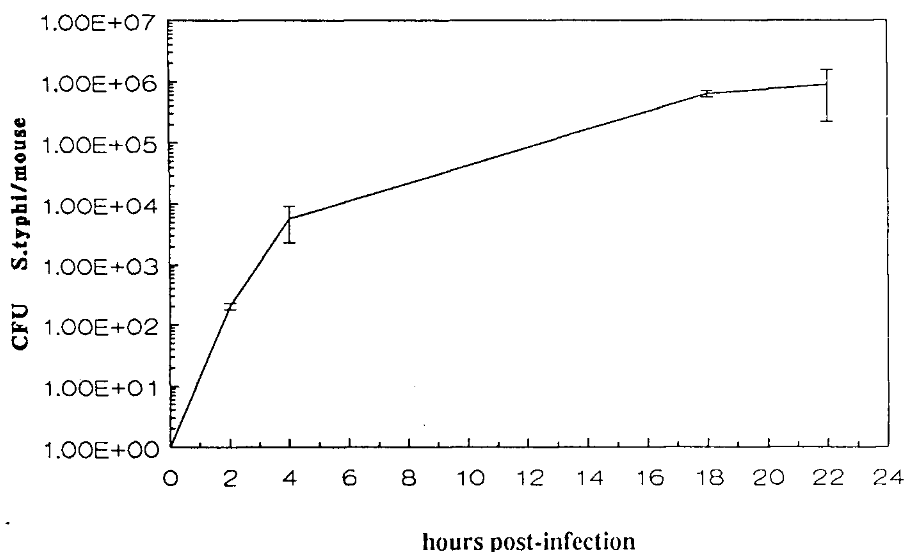

Fig. 1: Time course of changes in number of intracellular bacteria from peritoneal exudate cells (PEC) from mouse inoculated with *S. typhi* in mucin. Data, geometric means ± SDs of bacteria recovered from PEC obtained from 3 mice per group. CFU = colony forming units.

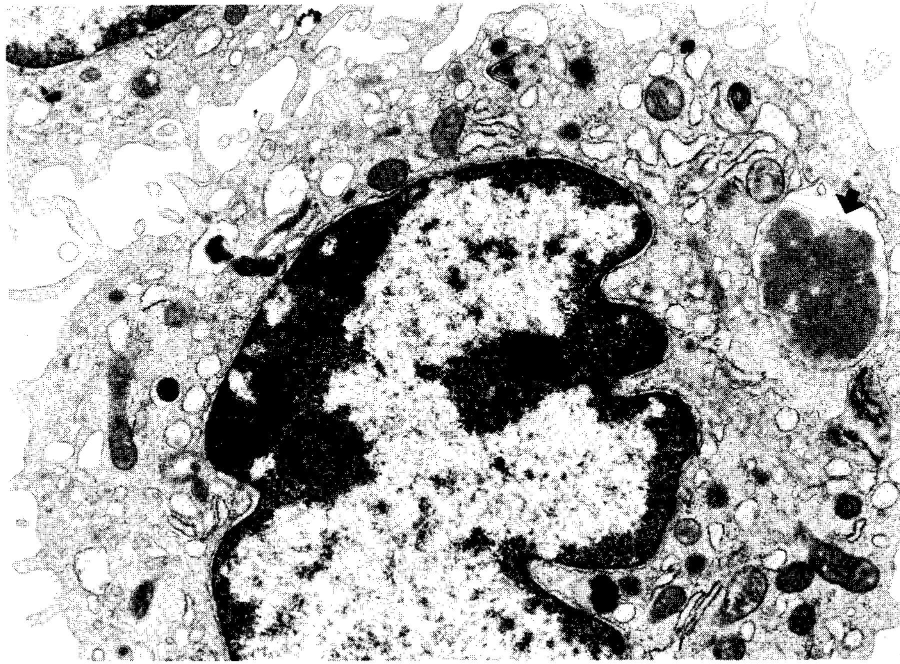

Fig. 2: A representative transmission electron micrograph of peritoneal exudate cells (PEC) from mice injected i.p. with mucin alone. At time 0, the cell exhibits a blistered cytoplasm. Arrow, electron dense material inside the vacuole. Magnification = 6.8 Kx

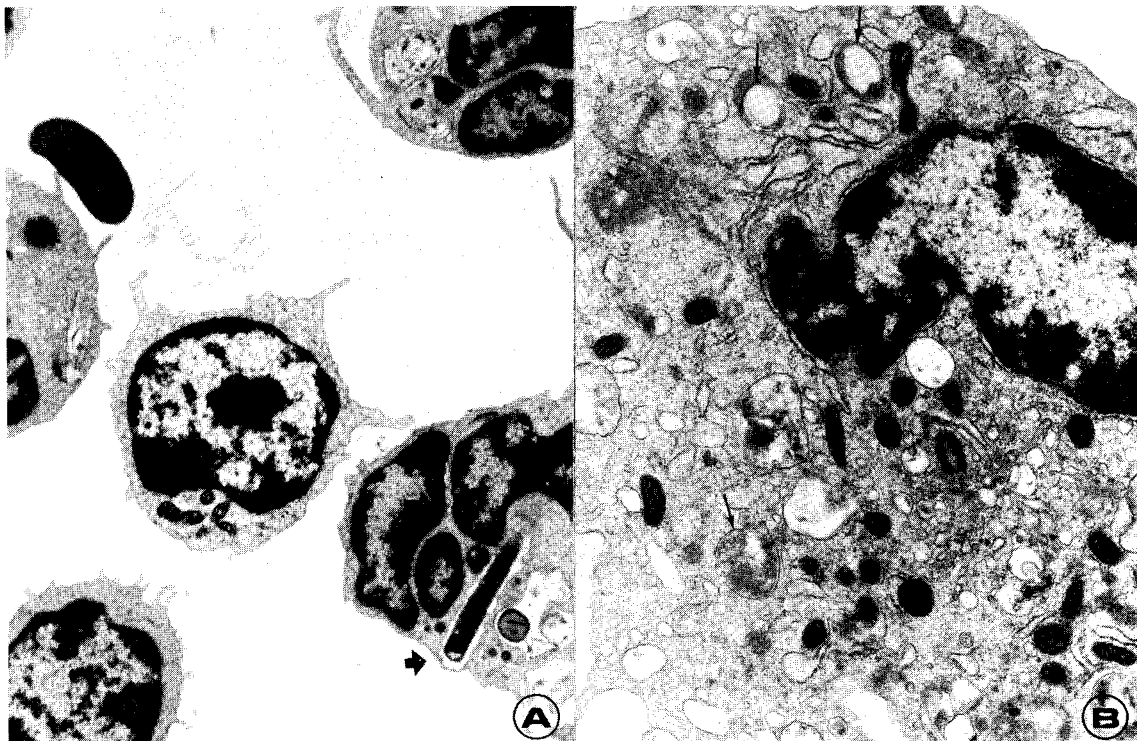

Fig. 3: Transmission electron micrographs of peritoneal exudate cells recovered from mice 4 h, after i.p. injections of: (A) *S. typhi* in mucin (thick arrow, intact bacteria in a phagolysosome); magnification = 3.9 Kx. (B) *S. typhi* in PBS (thin arrow, bacterial debris); magnification = 10 Kx.

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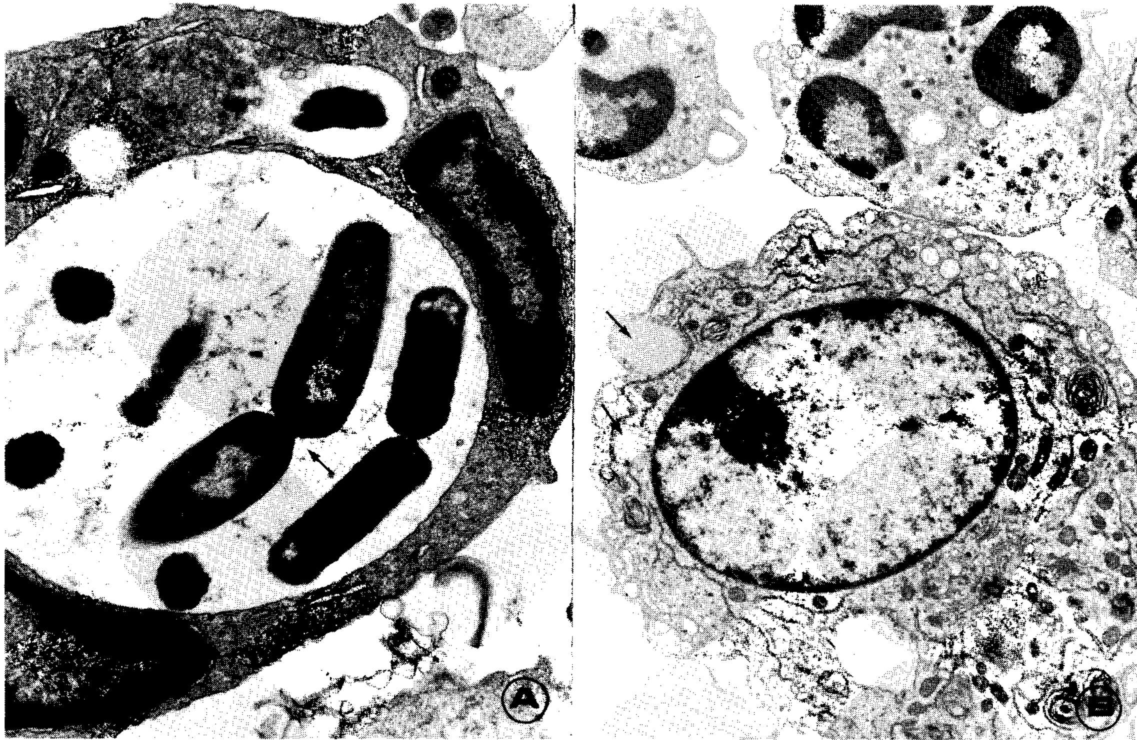

Fig. 4: Transmission electron micrographs of peritoneal exudate cells recovered from mice 15 h after i.p. injections of: (A) *S. typhi* in mucin (arrow, terminal state of multiplication of the bacteria); magnification = 10 Kx. (B) cells from animals injected with *S. typhi* in PBS (arrows, lipid droplets); magnification = 4.5 Kx.

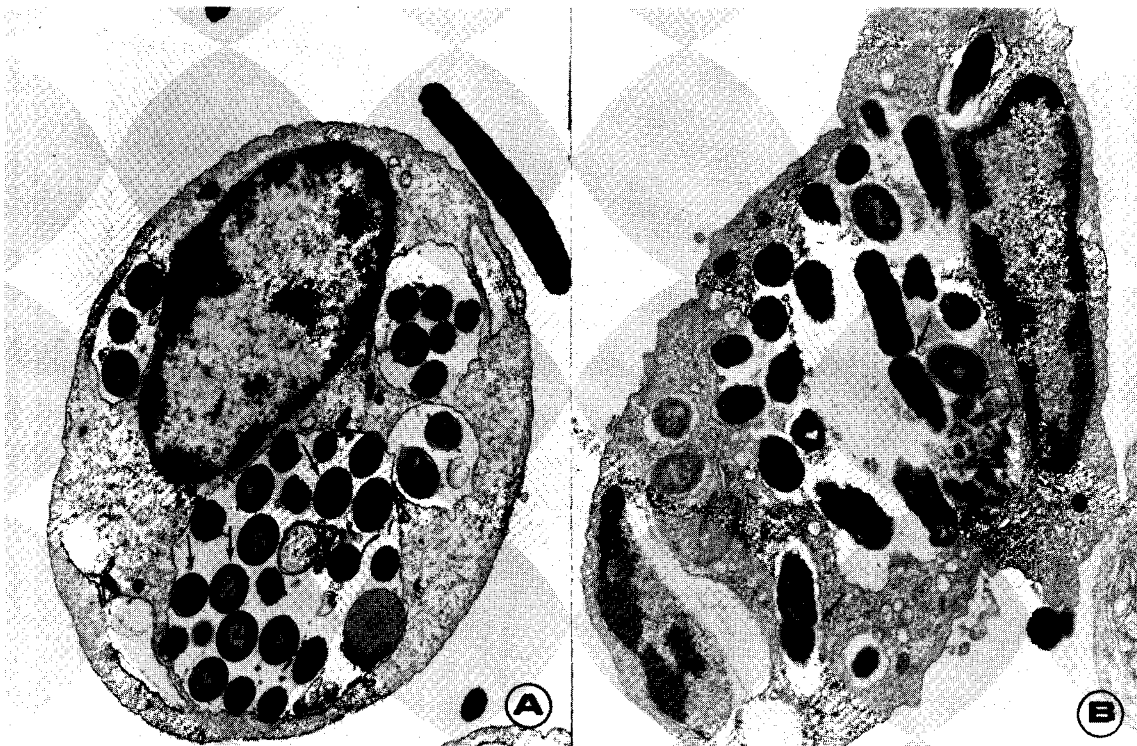

Fig. 5: Transmission electron micrographs of peritoneal exudate cells recovered from mice 22 h after i.p. injection of *S. typhi* in mucin. Large numbers of intact bacteria are seen. (A) arrows, well preserved bacteria within a phagolysosome; magnification = 5.6 Kx. (B) arrow, bacteria with septum, suggesting some bacterial replication; magnification = 6.8 Kx.

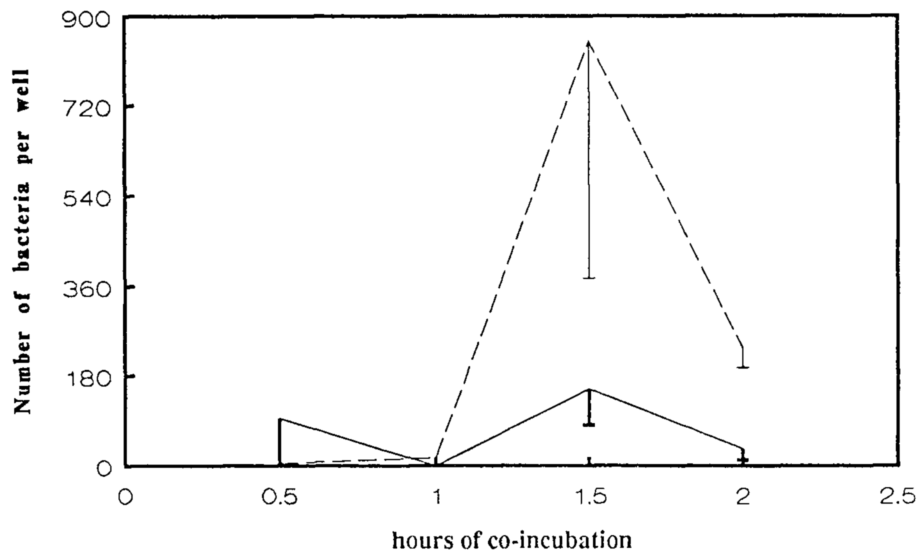

Fig. 6: Effect of co-incubation of murine J774.3 cell line with *S. typhi*, with or without mucin, on the recovery of viable bacteria from peritoneal exudate cells after culture in medium supplemented with gentamicin. Data, means–SDs of bacteria recovered from 3 micro wells. (——) without mucin. (- - -) with mucin.

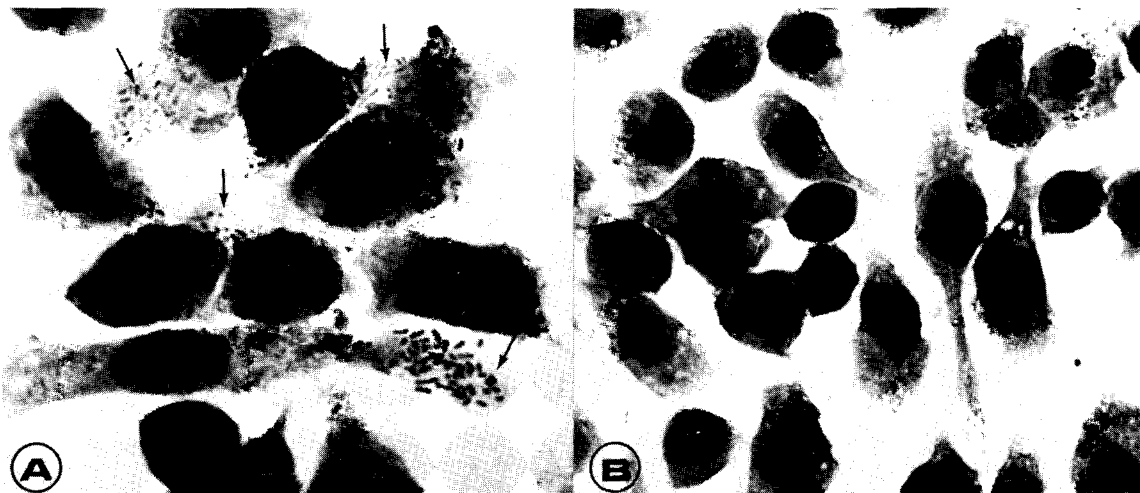

Fig. 7: Phase-contrast micrographs of murine J774.3 cell line. (A) cells infected with *S. typhi* in presence of mucin. Arrows, bacteria associated with cells. (B) Cells incubated with bacteria without mucin (control).

DISCUSSION

Our findings show that the intraperitoneal priming of mice with mucin renders the animals susceptible to *S. typhi* infection for two hours. The LD_{50} of 10^5 in Balb/c mice was higher than the value determined by Dougan *et al.* (1987) in the same strain of mice and identical to the LD_{50} determined by Isibasi *et al.* (1992) using NHI mice. Following removal of loose and cell-adhered bacteria by acid wash, viable bacteria were recovered from PEC. This suggested an intracellular location of bacteria, which was then confirmed by submitting the peritoneal exudate cells to TEM. Some extracellular growth of bacteria cannot be excluded in this model. The role of the extracellular growing bacteria is not clear because the onset of the septic shock occurs 46 hours after the virulence potentiating activity of mucin has disappeared. Furthermore TEM pictures of

PEC specimens, taken at different times after infection, indicate clearly a progressive increase in the number of well preserved intracellular bacteria inside large phagocytic vesicles. In animals inoculated with *S. typhi* in PBS without mucin, only bacterial debris were seen in PEC preparations.

In contrast to infection with *S. typhimurium* (O'Brien, 1982), a much higher dose of *S. typhi* is required to kill mucin primed mice. This may be due to the transient effect of mucin in enhancing virulence (Nungester *et al.*, 1936), hence, only a limited number of infective cycles may occur in the peritoneal cavity. A high initial dose of bacteria may be necessary to infect a large number of peritoneal cells, in order to trigger the septic shock several hours later. The difficulties encountered by some authors in observing intracellular bacteria at the terminal stages of experimental infections have led them to postulate that Salmonella species cannot survive and multiply inside phagocytic cells (Lin *et al.*, 1989; Hsu, 1989). However there is evidence of the opposite (Buchmeier and Heffron, 1991).

The mechanism by which porcine mucin enhances *S. typhi* virulence in mice remains to be elucidated. The large increase in the number of viable intracellular bacteria within vesicles of peritoneal phagocytic cells from mucin-treated animals and the observation that *in vitro* culture of the mouse reticuloendothelial sarcoma cell line J774.3 can be infected *in vitro* by *S. typhi* suspended in mucin, support the notion that mucin affects the intracellular digestion within the phagosomes, rather than interfering with phagocytocis, as suggested earlier by Nungester *et al.* (1936). However, these authors did not provide clear evidence about the intracellular location of bacteria. In addition, mucin can interfere with proteolytic digestion in phagolysosomes, because it is resistant to most common proteases (Marck and Sherman, 1991).

Mucin should also promote internalization of bacteria by a lectin-like activity, as proposed by Sharon (1984). Mucin may generate bacteria containing vacuoles on the PEC surface; subsequently, these vacuoles are internalized, dragging bacterial cells into the intracellular compartment.

Finally, the observation that Balb/c scid/scid is as resistant to *S. typhi* as its immunocompetent co-isogenic counterpart suggests that the intraperitoneal clearance of this bacterium relies mainly on the phagocytic activity. However, a role of natural killer (NK) cells in the innate immunity of Balb/c mice cannot be excluded. Balb/c scid/scid have normal NK cells (Dorhkind *et al.*, 1985), which could activate macrophages by the secretion of cytokines like IFN-γ and TNF-α, before the onset of the specific immune response, as described in the *S. typhimurium* infection of mice (Nauciel and Espinasse-Maes, 1992).

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