Role of previous stimulation on the neutrophil migration protects against the lethal effect of *Salmonella typhimurium*

Efeito da estimulação prévia sobre a migração de neutrófilos protege contra os efeitos letais da *Salmonella typhimurium*

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**SUMMARY**

The intraperitoneal inoculation of $3.8 \times 10^9$ CFU of alive *Salmonella typhimurium* caused 100% mortality in rats. In these animals a small number of neutrophils migrated to the peritoneal cavity. The intraperitoneal inoculation of the animals with thioglycollate (Tg, 90 mg), carrageenin (Cg, 500 mg), or killed *S. typhimurium* (KS, $1.9 \times 10^9$ CFU) 24 hours before challenge with alive *S. typhimurium* caused a significant neutrophil migration to the peritoneal cavity (determined 6 hours after challenge) and protected the animals against the lethal effect of the bacteria at a rate of 50%, 83% and 80%, respectively. However, similar treatment of the animals with brain heart infusion broth (BHI, *S. typhimurium* growth medium) protected the animals by only 23%. The migrating neutrophils in these animals represented less than 10% of those observed with other treatments. The phagocytosis activity was not associated with the survival rate as this activity was 2 times higher in the KS group than in the Tg and Cg groups, and the survival rate in this group was similar to that observed in Cg. These results suggest that the increase of neutrophil migration to the infectious site is an important factor associated with protection of the animals against the lethal effect of the bacterial infection.

**UNITERMS:** Migration; Neutrophils; *Salmonella typhimurium*; Peritonitis; Septicemia; Phagocytosis.

**INTRODUCTION**

Several studies have established that Gram-negative bacteremia or circulating endotoxin decreases the ability of neutrophils to migrate to inflammatory sites, a phenomenon which may play an important role in the evolution of septicemia. It has been demonstrated that neutrophil migration impairment is mediated by inhibitory factors, including interleukin-8, tumour necrosis factor, and a factor named neutrophil recruitment inhibitory factor. Similarly to endotoxin, intravenous administration of these cytokines inhibited neutrophil migration induced by different inflammatory stimuli. The pathophysiological events associated with neutrophil migration, such as exudation, are also inhibited by the treatment of the animals with these cytokines. Neutrophil inhibitory factor has also been found to be associated with other illnesses, such as diabetes mellitus and lepromatous leprosy.

Despite the demonstration that the blockage of neutrophil migration contributes to evolution to septicemia evolution, there is no clear evidence that the reestablishment of the neutrophil migration to the infectious site protects animals against the lethal effect of bacterial infection. In the present investigation we demonstrated that restoration of neutrophil migration to the infection site protected rats against the lethal effect of *Salmonella typhimurium* infection.

**MATERIAL AND METHOD**

**Animals**

Male Wistar rats weighing 180-200 g were used. Animals were housed in temperature-controlled rooms and received water and food *ad libitum*.

**Preparation of bacteria**

The bacterial strain used in this study was isolated from a septicemic outbreak guinea pig and was identified as *Salmonella typhimurium*. To increase its virulence to Wistar rats, ten successive inoculations into the peritoneal cavity ($3.8 \times 10^9$ CFU) were necessary (100% mortality). The bacteria
were then harvested, grown on brain heart infusion broth (BHI), lyophilized and stored at 4°C. For use, the lyophilized bacteria were grown for 24 hours at 37°C in 50 ml BHI broth.

Experimental design

Rats were divided into six experimental groups: naive, control (N group); treated with intraperitoneal injection of 1 ml of BHI containing 1.9 x 10⁹ CFU of S. typhimurium killed by boiling in water for 30 min. (KS group); treated with intraperitoneal injection of 2 ml sterilized BHI broth (BHI group); received intraperitoneal injection of 3 ml thioglycollate solution (3% w/v, 90 mg/cavity; Tg group); treated with intraperitoneal injection of 500 µg of carrageenin (Cg group). All these groups were challenged 24 or 96 hours later with an intraperitoneal injection of 2 ml BHI containing 3.8 x 10⁹ CFU of alive S. typhimurium. Another group received only the alive S. typhimurium challenge (Ls group).

In another assay with naive, KS and Ls groups the treatments and the challenge 24 hours later were done in the pleural cavity.

Determination of the number of cells in the peritoneal and pleural cavities

Twenty four or ninety six hours after the intraperitoneal injections with Cg, Tg, KS and BHI or 6 hours after alive S. typhimurium challenge, animals were sacrificed. For the pleural assays the animals were treated by 24 hours and challenged by 6 hours when were slaughtered. Their peritoneal and pleural cavities washed with PBS (10 and 5 ml, respectively) containing EDTA (10% v/v, pH 7.4). The peritoneal or pleural fluid was collected with Pasteur pipettes, placed in conical tubes and centrifuged at 2,000 rpm for 10 min. in a clinical centrifuge. The cells were then resuspended in 2.0 ml heparinized (5 IU/ml) and the suspension was diluted 1:20 in Turk fluid in white cell pipettes for total leucocyte counts. Neutrophil migration was performed on panchromatically stained smears.

Leukogram assay

Blood samples were taken from the retroorbital plexus of the rats before and after intraperitoneal treatments. The S. typhimurium challenge was done 24 hours after the treatments. Total and differential cell counts were performed and the results are reported as the number of neutrophils per ml of blood.

Phagocytosis activity of neutrophils

Six hours after S. typhimurium challenge, animals were exsanguinated and their peritoneal cavities washed with 10 ml PBS containing EDTA (10% v/v, pH 7.4). Total and differential cells counts were performed and, in the smears used for differential cell count, the percentage of the neutrophils that phagocytosed at least 3 bacteria was also determined. Results are reported as the total number of neutrophils in phagocytosis activity per cavity.

Survival rate

Survival rate of rats 48 hours after the S. typhimurium challenge was also determined in each experimental group.

Statistical analysis

Results are reported as the means ± standard error of the mean (SEM). Analysis of variance and Tukey’s test were used for comparison of means. Statistical differences were considered to be significant at p < 0.05.

RESULTS

Preliminary experiments showed that intraperitoneal administration of 1.9, 3.8 or 7.6 x 10⁹ CFU of killed S. typhimurium or 1.9 x 10⁹ CFU of alive S. typhimurium did not kill the animals. However, the administration of 3.8 x 10⁹ CFU of alive S. typhimurium resulted in 100% mortality 24 hours after. Previous administration of killed bacteria (1.9 x 10⁹ CFU) 24 hours before the challenge with alive bacteria protected 80% of the animals. The number of neutrophils that emigrated to peritoneal cavities 6 hours after the challenge was 58.9 ± 5.3 x 10⁶/mm³. In animals that were not treated with the killed bacteria, the number of neutrophils that emigrated after intraperitoneal injection of 1.9 or 3.8 x 10⁹ CFU of alive S. typhimurium were 22.1 ± 7.1 x 10⁶/mm³ and 3.5 ± 0.9 x 10⁶/mm³, respectively, a significant reduction (p < 0.05).

Peritoneal cell counts after treatments

Fig. 1 shows that 24 hours (1A) and 96 hours (1C) after the intraperitoneal treatment of the animals with BHI, Tg, KS or Cg occurred a neutrophil accumulation in the peritoneal cavities of the animals. Neutrophil migration was predominant in the Cg group. In the Tg and Cg groups a migration of mononuclear cells was also observed. The number of eosinophils in all experimental groups did not differ from that of the control. Fig. 1B shows that the challenge of animals with alive S. typhimurium promoted a significantly (p < 0.05) increase in the number of neutrophils accumulated in the Tg and KS groups after 6 hours. In the Cg group, the number of neutrophils was similar to that present before the S. typhimurium challenge (Fig. 1A). In the BHI group, the number of neutrophil migrated to the peritoneal cavity after the challenge has an increase statistically significant (Fig. 1B) but not as massive as in the Tg, KS or Cg group that was similar to that present before the S. typhimurium challenge (Fig. 1A). In the LS group neutrophil migration after the inoculation was not significant (Fig. 1B). In all groups the number of eosinophils after the challenge was similar to that observed before the challenge. The number of mononuclear

Leukocyte numbers in the peritoneal cavities of rats 24 (panel A) or 96 (panel C) hours after indicated treatments and another groups that received the same treatments and 6 hours after S. typhimurium challenge (panel B and D). The animals were treated with killed S. typhimurium (KS, 1.9 x 10^9 CFU); BHI broth (BHI, 1 ml); thioglycollate (Tg, 90 mg) and carrageenin (Cg, 500 µg) and challenged with alive S. typhimurium (3.8 x 10^9 CFU). The first set of bars represents the resident peritoneal cells in naive animals (N). The number of animals per group is indicated inside the squares. Results are expressed as means ± SEM for six to seventeen animals/group. * represents significant differences compared to respective controls (p < 0.01, Tukey’s test).

Figure 1

Leukocyte numbers in the peritoneal cavities of rats 24 (panel A) or 96 (panel C) hours after indicated treatments and another groups that received the same treatments and 6 hours after S. typhimurium challenge (panel B and D). The animals were treated with killed S. typhimurium (KS, 1.9 x 10^9 CFU); BHI broth (BHI, 1 ml); thioglycollate (Tg, 90 mg) and carrageenin (Cg, 500 µg) and challenged with alive S. typhimurium (3.8 x 10^9 CFU). The first set of bars represents the resident peritoneal cells in naive animals (N). The number of animals per group is indicated inside the squares. Results are expressed as means ± SEM for six to seventeen animals/group. * represents significant differences compared to respective controls (p < 0.01, Tukey’s test).

Moreover, when the challenge was done 96 hours (Fig. 1D) after the treatments with Tg, Cg and KS, the number of mononuclear cells in the peritoneal cavity before the challenge (Fig. 1C) was lower than that observed when the interval was 24 hours, being 27%, 60% and 1%, respectively. In these groups, reduction in neutrophil migration 6 hours after the challenge with alive salmonella was also observed (Fig. 1D). Compared to the 24-hours treatment the reduction was of 55%, 74% and 32% in the Tg, Cg, and KS groups, respectively.
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Table 1
Phagocytosis activity of the migrating neutrophils and survival rate of the animals in the different experimental groups. Jaboticabal, SP - 1994.

The number of migrating neutrophils and phagocytosis activity (absolute number and percentage) were determined 6 hours after alive S. typhimurium challenge. The survival rate was determined during 24-hours period after challenge. The results are expressed as means ± SEM, and the number of animals per group is given in parenthesis. * represents significant differences compared to respective controls (p < 0.01, Tukey’s test).

Table 2
Number of circulating neutrophils before and at different times after ip treatment with killed S. typhimurium (KS, 1.9 x 10⁹ CFU); thigoligolate (Tg, 90 mg) and carrageenin (Cg, 500 µg). The challenge was done with alive S. typhimurium (LS, 3.8 x 10⁹ CFU) 24 hours after the treatments. The animals were slaughtered six hours after challenge (48 hours after the previous treatments) or died before. The results are expressed as mean ± SEM x 10³/mm³. In naive rats the results of the circulating neutrophils counts at the same times were: 0 = 1.75 x 10⁹/mm³; 1h = 2.2 x 10⁹/mm³; 2h = 1.75 x 10⁹/mm³; 4h = 2.9 x 10⁹/mm³; 6h = 3.74 x 10⁹/mm³; 24h = 3.1 x 10⁹/mm³. Jaboticabal, SP – 1994.

Pleural cell counts
The number of neutrophils that migrated into pleural cavities 6 hours after intrapleural injection of 3.8 x 10⁹ CFU of alive S. typhimurium was 2.90 ± 0.3 x 10³/mm³ washed fluid and 100% of the animals died within 24 hours after the challenge. However, if the animals were treated 24 hours before with 1.9 x 10⁹ CFU of killed S. typhimurium (KS) the number of neutrophils that migrated into the pleural cavities was 44.5 ± 0.3 x 10³/mm³ washed fluid and only 33% of the animals died. In these animals a significant accumulation of mononuclear cells was observed at the moment of the challenge (naive animals: 13.9 ± 0.2 x 10³ cells/mm³; KS,0 group: 28 ± 2 x 10³ cells/mm³ washed fluid; n = 6).

Phagocytosis of S. typhimurium by neutrophils and animal survival rate
The number of neutrophils in phagocytosis activity was significantly increased in the Tg, KS and Cg groups, compared to the LS group. In the KS group, the number of neutrophils that phagocytosed bacteria was 2 times that seen in the Tg or Cg group. As a consequence of the low number of migrating neutrophils, in the LS and BHI groups the number of neutrophils that phagocytosed bacteria was also non-significant. However, the challenge with alive bacteria did not change the ability of the migrating neutrophils to phagocytose bacteria, since the percentages of phagocytosis in the BHI, KS, Tg, Cg and LS groups were 60.8%, 75.5%, 49.9%, 52% and 100%, respectively (Tab. 1). Animal survival rates were high in the Tg (57%), KS (83%) and Cg (80%) groups. In the LS and BHI groups, animal survival rates 24
hours after the challenge were only 0% and 23%, respectively (Tab. 1). Most of the animals died within 6-14 hours after the bacterial challenge. In the other groups, the animals that survived were sacrificed 3 days after the challenge.

During 96 hours interval between treatment and challenge with alive bacteria the animal survival rates in the Tg, Cg and KS groups were 25%, 25% and 50%, respectively. These values represented a reduction in the survival rate of 55%, 69% and 40%, respectively, compared to the control 24-hours pretreatment treatment (data not shown). Moreover, the simultaneous administration of Cg and alive bacteria did not cause neutrophil migration (determined 6 hours after the bacterial challenge) and did not protect animals from death (data not shown).

**Circulating neutrophils**

Tab. 2 shows that the treatment of the animals with BHI broth, thioglycollate, carrageenin and killed salmonella promoted an increase in the number of circulating neutrophils within the first 6 hours, returning to control levels 24 hours after, except in the KS group. In this group neutrophilia persisted until challenge time. The *S. typhimurium* challenge induced a transient neutropenia in all groups.

**DISCUSSION**

The inhibition of neutrophil migration has been demonstrated during Gram-negative bacteremia or in the presence of circulating endotoxin. This effect is thought to play an important role in the evolution of sepsis. This inhibition may be mediated by cytokines, such as TNF-α, IL-8 and an unpurified cytokine named NRIF released by the host cells, mainly mononuclear cells. Extending these studies, we showed in the present investigation that when the neutrophil migration occurs there was a proportional protection of the rats against the lethal effect of *S. typhimurium* peritonitis. It was observed that the intraperitoneal inoculation of 3.8 x 10^9 CFU of alive *S. typhimurium* killed the rats by 100%. In these animals, a reduced number of neutrophils migrated to the peritoneal cavity. However, if the dose of alive salmonella injected was 1.9 x 10^9 CFU, the animals did not die and a significant number of neutrophils migrated to the peritoneal cavity. The role of TNF-α, IL-8 and NRIF in neutrophil migration impairment observed after challenge with alive *S. typhimurium* is under investigation.

Moreover, treatments of the animals with BHI, Tg, KS and Cg, 24 hours prior to challenge, induced different levels of protection: 23%, 57%, 80%, and 83%, respectively. It is important to note that, when the number of migrating neutrophils to the site of bacterial challenge was high (Tg, KS and Cg), survival rate was also high. In the Tg and KS groups, most neutrophils migrated after the bacterial challenge, while in the Cg group, neutrophils were already present in the peritoneal cavity at challenge time, without a further increase in number. Similar to the LS group, neutrophil accumulation in the BHI group was negligible and protection was low. Confirming that protection of the animals depends of the number of migrating neutrophils, it was observed that when the challenge was given 96 hours after treatment the number of recruited neutrophils and survival rate were lower than those observed for 24-hours treatments. The protection phenomenon is not specific for the peritoneal cavity since the protection pattern was similar when the treatment and challenge were done in the pleural cavity. Although the mechanism by which the treatments promoted a proportional neutrophil migration caused by the alive *S. typhimurium* challenge was not investigated. We suggest that these treatments increase the capacity of the resident peritoneal cells to release neutrophil chemotactic factors after the bacterial challenge. The increase in the amount of chemotactic factors released may overshadow the effect of the neutrophil inhibitory factor released after alive *S. typhimurium* challenge. It has been demonstrated that the rise in the number of mononuclear cells by previous administration of Tg causes a proportional increase in neutrophil migration induced by different stimuli such as Cg, LPS or zymosan. In the Tg and Cg groups, an increase in the number of mononuclear cells in the peritoneal cavity 24 hours before the challenge was observed in the present experiment. In the KS group, in which the treatment did not increase the number of macrophage, the treatment (killed *S. typhimurium*) possibly primed the resident cells which may in turn release more efficiently chemotactic factors. There is evidence that primed mononuclear cells release more cytokines. Alternatively, as discussed below, neutrophilia observed after the injection of killed bacteria may contribute to the high number of migrating neutrophils.

The number of neutrophils in phagocytosis activity in the peritoneal cavities of animals from the KS group was higher than those observed in the Tg or Cg group. Furthermore, the survival rate in the KS group did not differ from that observed in the Cg group, suggesting that either in the Cg group the microbicidal activity of neutrophils is enhanced or different microbicidal mechanisms are involved in these groups. In the BHI and LS groups, the number of neutrophils in phagocytosis activity was low because the number of migrating neutrophils was also non-significant. It is important to point out that in the LS group all the migrating cells phagocytosed bacteria. This fact suggests that the infection did not affect the phagocytosis process, and reinforces the concept that the main problem in septicemia is the impairment of neutrophil migration. This fact may exert a facilitatory effect on the bacteria multiplication and its organic diffusion in the host.

To investigate if a systemic effect could also be involved in the protection process, the animals were treated with KS in the peritoneal cavity and challenged with alive bacteria in the
pleural cavity, or treated in the pleural cavity and challenged in the peritoneum. With this approach, the survival rate was only 30%. This fact suggests that protection caused by treatment is mainly a local phenomenon, dependent on the neutrophil migration. However, a marginal contribution of systemic mechanisms is also involved. Systemic unspecific protection against the lethal effect of *Klebsiella pneumoniae*, *Trypanosoma cruzi*, and *Candida albicans* has been previously reported in the literature, and macrophages had an important role in these cases."

"Except for the KS group, in all other groups an increase in circulating neutrophils occurred within the first 4 hours following the previous treatment, returning to basal values 24 hours later. In the KS group, neutrophilia remained unchanged until challenge time, decreasing drastically after 1 hour later and was followed by an increase in the number of neutrophils in the peritoneal cavity after 6 hours. This fact suggests that these circulating cells migrated from the vascular compartment to the inflammatory focus and may explain the higher migration observed in this group.

In summary, our results suggest that the neutrophil migration to the infectious inflammatory focus is an important procedure to protect the host against the lethal effect of the bacterial infection.

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RESUMO

A inoculação intraperitoneal de 3,8 x 10⁶ CFU de *Salmonella typhimurium* viva causou 100% de mortalidade em ratos. Nesses animais, pequeno número de neutrófilos migraram para a cavidade peritoneal. A injeção intraperitoneal de tioglicolato (TG, 90 mg), carragenina (Cg, 500 mg) ou salmonelona morta pelo calor (KS, 1,9 x 10⁹ CFU) 24 horas antes do desafio com *S. typhimurium* viva causou maior (p < 0,01) migração de neutrófilos para a cavidade peritoneal após 6 horas e conferiu proteção aos animais contra os efeitos letais da bactéria que foram da ordem de 50%, 83% e 80%, respectivamente. Todavia, o tratamento semelhante dos animais com o meio de cultivo "infusão de cérebro e coração" (brain heart infusion-BHI) estéril para o sítio infeccioso induzida pelas administrações prévias das diferentes substâncias é um importante fator associado com a proteção dos animais contra os efeitos letais da infecção bacteriana.

UNITERMOS: Migração; Neutrófilos; *Salmonella typhimurium*; Peritonite; Septicemia; Fagocitose.

REFERENCES


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