Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmacology 525 (2005) 161–169
www.elsevier.com/locate/ejphar

Effect of mast cells depletion on the failure of neutrophil migration during sepsis

Michel Carvalho [a], Cláudia Benjamim [a], Fabrício Santos [b], Sérgio Ferreira [a], Fernando Cunha [a,*]

[a] Department of Pharmacology, Faculty of Medicine Ribeirão Preto, University of São Paulo, São Paulo, Brazil
[b] Department of Health, University of Santa Cruz, Ilhéus, Bahia, Brazil

Received 4 July 2005; received in revised form 15 September 2005; accepted 27 September 2005
Available online 2 November 2005

Abstract

The possible role of mast cell in neutrophil migration failure during sepsis was examined in a polymicrobial sepsis model in mice. Mast cells were depleted by compound 48/80 or lysed by distilled water, both preventing the neutrophil migration failure. This phenomenon was accompanied by reduction of bacteria in the peritoneal cavity and blood, serum tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β) and nitrate (NO_3) and by an increase in mice survival rate. Neither neutrophil migration failure nor significant mortality was observed when lethal inoculum was injected into the air-pouch model, a cavity poorly populated by mast cells. Confirming that neutrophil migration failure is a phenomenon induced by systemic circulating mediators, it was observed that i.p. administration of lethal inoculum induced a neutrophil migration failure to the air pouch inoculated with non-lethal bacterial challenge. These results suggest that mast cells have a key role in the genesis of neutrophil migration failure, and, consequently, contribute to the systemic inflammatory response and mortality in severe sepsis.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Sepsis; Mast cell; Neutrophil migration; Compound 48/80

1. Introduction

Despite extensive research over the last few decades, sepsis remains the main cause of death in intensive care units, with mortality rates between 30% and 70% (Riedemann et al., 2003). Sepsis is defined as a systemic inflammatory response and is the consequence of the inability of the organism to restrict locally an infection. Spreading of the infection induces a systemic release of inflammatory mediators such as tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), IL-6, IL-10, eicosanoids, nitric oxide (NO) and activated complement system. These mediators are responsible for most manifestations of sepsis, i.e., cardiovascular failure, leukocyte dysfunction and coagulation disorders. Indeed, the overproduction of pro-inflammatory mediators is a critical determinant in the development of sequential multiple organ failure, septic shock and death (Cohen, 2002; Walley et al., 1996). Although several anti-inflammatory strategies in clinical trials for sepsis treatment have been used, such as antibodies against cytokines and eicosanoids antagonists, none of them has been successful so far (Patel et al., 2003). A critical analysis of the literature indicates that the failure of anti-inflammatory approaches in sepsis could be a consequence of an inhibition of the local inflammatory response, such as the neutrophil migration to the infectious focus, allowing enhancement of bacteria spreading.

In fact, neutrophil migration to the infectious focus is extremely important for local control of bacterial growth and consequently for the prevention of bacterial dissemination (Verdrengh and Tarkowiski, 1997). Recently we demonstrated that failure of neutrophil migration is observed in lethal sepsis induced by cecal ligation and puncture or *S. aureus* inoculation. In these lethal models, neutrophil migration at the site of infection was accompanied by an increased number of bacteria in peritoneal fluid and blood and by a high mortality rate. Conversely, in non-lethal sepsis, in which the neutrophil migration failure was not observed, the bacterial infection was restricted to the peritoneal cavity, with the animals exhibiting a low mortality rate (Benjamim et al., 2002; Crossara-Alberto et

* Corresponding author. Faculdade de Medicina de Ribeirão Preto-USP Departamento de Farmacologia Laboratório de Inflamação Av. Bandeirantes, 3900, CEP 14049-900 Ribeirão Preto-SP Brazil. Tel.: +55 16 6023205; fax: +55 16 6332301.
E-mail address: fdqcunha@fmrp.usp.br (F. Cunha).

0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2005.09.049

al., 2002). Furthermore, reduction in the ability of neutrophil chemotaxis was also observed in patients with severe sepsis (Tavares-Murta et al., 2002).

Although the essential contribution of resident phagocytes present in the infectious focus to sepsis resolution has been described (Topley et al., 1995), the role of mast cells remains controversial. According to Echtenacher et al. (1996) and Malaviya et al. (1996), mast cell-deficient mice (W/W^v) are more susceptible to death than syngeneic wild-type animals in sepsis models involving cecal ligation and puncture or *Klebsiella pneumoniae*. The higher susceptibility of W/W^v mice was correlated with their inability to release TNF-α in the infectious focus. In fact, the W/W^v animals' survival rate could be increased by reconstitution of the peritoneal cavity with mast cells obtained from wild-type mice or by i.p. administration of exogenous TNF-α. Furthermore, it was also demonstrated that release of TNF-α by mast cells plays an important role in the recruitment of neutrophils into the peritoneal cavity (Malaviya et al., 1996).

On the other hand, there are also evidences that mast cell mediators including histamine and dipeptidyl peptidase I contribute to death of septic host (Mallen-St Clair et al., 2004; Hori et al., 2002). Furthermore, Hirasawa et al. (2002) have shown that histamine down-regulates the neutrophil infiltration in allergic inflammation.

Thus, taking into account that mast cells are a relevant source of inflammatory and chemotactic mediators, our aim was to investigate the role of these cells in the establishment of neutrophil migration failure in a live bacteria inoculation model and its relevance for severe sepsis outcome. Our results demonstrate that during lethal sepsis, mast cells have a deleterious role, inducing an overwhelming release of cytokines and NO, and, therefore, contributing to neutrophil migration failure and animal mortality.

2. Materials and methods

2.1. Mice

For all experiments, male Swiss mice (weight 24–28 g) were used. The animals were housed in cages in temperature-controlled rooms (22–25 °C) and received water and food ad libitum. All experiments were conducted according to the guidelines of the ethics committee of the School of Medicine of Ribeirão Preto, University of São Paulo, São Paulo, Brazil. Each experiment used 4–5 mice and was repeated 2–3 times.

2.2. Depletion of mast cells

Mice were pretreated i.p. every 12 h for four days, with the mast cell degranulator compound 48/80 dissolved in PBS (phosphate-buffered saline) and injected at 200 μl/cavity according to the following schemes: 1st day, 0.6 mg/kg; 2nd day, 1.0 mg/kg; 3rd day, 1.2 mg/kg; 4th day, 2. 4 mg/kg. The induction of sepsis was performed 24 h after the last dose of compound 48/80 (Ribeiro et al., 1991).

In other set of experiments to deplete the peritoneal mast cells, the animals were injected i.p. with 2 ml of sterile distilled water 48 h before the administration of the bacterial inoculum (Medonça et al., 1986).

2.3. Sepsis induced by bacteria inoculation

The cecum contents of three mice were removed by laparotomy, diluted in PBS and filtered through sterile gauze. An aliquot of cecal content suspension was diluted in brain and heart infusion medium (BHI medium) (Difco Laboratories, Detroit) and incubated 18 h at 37 °C. The culture in BHI medium was repeated twice to obtain a greater amount of bacteria. The final suspension was centrifuged (10 min; 1240 *g*) and washed twice with PBS. The bacterial suspension was lyophilized (Hetovac, mod. CT 110) and the tubes containing this were stored at −70 °C. All steps were performed in sterile conditions. For determination of the number of bacteria, the lyophilized contents of three tubes were diluted each one in 50 ml of BHI medium, homogenized and incubated 18 h at 37 °C. Next, the bacterial suspension was centrifuged (10 min; 1240 *g*), washed twice with PBS and the final volume was diluted in 10 ml of PBS. The number of colony forming units (CFU) of the bacteria in the suspension was determined through serial log dilutions and plating on Mueller–Hinton agar dishes (Difco Laboratories, Detroit, USA); CFU were counted after 18 h and the results were expressed as the number of CFU per ml. For induction of sepsis, 200 μl of the final suspension with the volume adjusted to contain the desired number of bacteria, was injected into the peritoneal cavity.

2.4. Air-pouch model

The procedure to obtain the air-pouches was previously described by Edwards et al. (Edwards et al., 1981). Briefly, 5 ml of sterile air were injected s.c. in the back of the mice. Three days later, the same procedure was repeated by injecting 3 ml of sterile air to maintain the cavity patent. The animals were inoculated with bacteria two days later.

2.5. Neutrophil migration into the peritoneal cavity and air-pouch

Neutrophil migration was quantified at 6 h after bacterial inoculation. The animals were killed and the cells from peritoneal and air-pouch cavities were collected by washing the cavities with 3 ml of sterile PBS containing 1 mM of EDTA. Total counts were made in a cell counter (Coulter Ac T) and differential cell counts were made on centrifuge slides (Cytospin 3, Shandon Southern Products, Atsmoore, UK) stained by the May–Grunwald–Giemsa method. The results were expressed as the number of neutrophils per cavity.

2.6. Bacterial count in blood, peritoneal and air-pouch cavities

Aliquots of serial dilutions of ten microliters of blood, peritoneal and air-pouch lavages were plated on Mueller–

Hinton agar dishes and incubated at 37 °C; colony-forming units were analyzed after 18 h and the results were expressed as the number of CFU per ml.

2.7. Cytokine determination

The serum concentrations of TNF-α, IL-1β, and IL-10 were determined by a double-ligand ELISA (enzyme-linked immunosorbent assay). Briefly, flat-bottomed 96-well microtiter plates were coated with 100 μl per well of antibody specific to one of the above cytokines at a dilution of 2 μg/ml (TNF-α and IL-1β) and 1 μg/ml (IL-10) in coating buffer and incubated overnight at 4 °C. Next, the plates were washed and non-specific binding was blocked for 120 min at 37 °C with 1% bovine serum albumin. Samples (undiluted) and standards were loaded on to plates. Recombinant murine TNF-α, IL-1β and IL-10 standard curves were used to calculate the cytokine concentrations. The plates were thoroughly washed and the previously titrated biotinylated polyclonal or monoclonal anticytokine antibody was added. After 1 h avidin–peroxidase (diluted 1 : 5000) was added to each well for 15 min and each plate was thoroughly washed again. Next, substrate (4 mg of o-phenilenediamine diidrochlorite + 0.4 μl of H_2O_2 in 1 ml of substrate buffer) was added and the reaction was stopped with H_2SO_4 (1 M). The optical density was measured on an ELISA plate scanner (Spectra Max 250 – Molecular Device) at 490 nm. The results were expressed as ng of TNF-α, IL-10 and IL-1β /ml of serum, comparing the optical density in the samples with the standard curves.

2.8. Determination of serum nitrate concentration

Retro-orbital blood was collected in anticoagulant-free tubes for serum nitrate assay. Blood samples were centrifuged at 180 × *g* for 15 min and the sera were stored at −20° until required. The nitrate concentration was determined enzymatically by reducing nitrate with nitrate reductase. Briefly, 50 μl of nondiluted serum samples were incubated with the same volume of reductase buffer (0.1 M potassium phosphate, pH 7.5, containing 1 nM nicotinamide adenine dinucleotide phosphate, 10 mM flavin adenine dinucleotide phosphate, 10 mM flavin adenine dinucleotide, and 4 units of nitrate reductase/ml) for 20 h at 37 °C. A standard nitrate curve was obtained by incubating sodium nitrate (10–200 μM) with the reductase buffer. The total amount of nitrite was determined by the colorimetric Griess method. Briefly, the samples were incubated with the same volume of freshly prepared Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid). Absorbance at 550 nm was determined by using a multiwell plate reader (Multiskan MCC/340 MKII, Flow Laboratories). The results are reported as micromol (μM) of $NO_3 + NO_2$.

2.9. Drugs, reagents and antibodies

The following materials were obtained from the sources indicated. rmIL-1β (lot 63/668; specific activity: 100 000 IU/ 0.1 μg ampoule), rmTNF-α (lot 99/532; specific activity: 200 000 IU/1 μg ampoule), purified anti-mouse TNF-α, biotinylated anti-mouse TNF-α (lot 250697) and biotinylated anti-mouse IL-1β (lot 250 997) were gifts of Dr. Steve Poole (National Institute for Biological Standards and Control, London, UK). rmIL-10 (417-ML), anti-mouse IL-10 monoclonal antibody (MAB417) and biotinylated anti-mouse IL-10 (BAF417) were purchased from R and D Laboratories (Minneapolis MN, USA). Compound 48/80 and all other reagents were purchased from Sigma Co. (USA).

2.10. Statistical analysis

The data (except survival curves) are reported as mean ± S.E.M. The experiments were repeated at least two or three times. Analysis of variance was used to compare the means of different treatments. If significance was identified, individual comparisons were subsequently made by Bonferroni's *t*-test for unpaired values. Statistical significance was set at $P < 0.05$. Survival rates were expressed as percentages, and a log rank test (x^2 test) was used to examine differences between survival curves.

3. Results

3.1. Mortality and neutrophil migration following bacterial inoculation

Fig. 1A shows the survival curves of mice injected with different polymicrobial inocula (1.0×10^4; 1.5×10^4; 2.5×10^4; 1.0×10^5 and 2.0×10^5 CFU/cavity). All of the animals inoculated with 1.0×10^4 and 1.5×10^4 CFU/cavity survived for at least 5 days after bacterial challenge. The animals inoculated with 2.5×10^4 showed 20% mortality at day two, whereas animals inoculated with 1.0×10^5 and 2.0×10^5 CFU/cavity already showed 100% mortality on the second day of observation. All mice that were subject to lethal inoculation developed early clinical signs of sepsis, including lethargy, piloerection and tachypnea. The animals injected with non-lethal inoculum also develop early clinical signs but the manifestations disappeared within the first 24 h.

Fig. 1B shows that neutrophil migration into the peritoneal cavity of animals injected with increasing inocula followed a bell-shaped curve. The neutrophil migration increased significantly after intraperitoneal (i.p) injection of the 10^4 bacteria, peaked with the inoculum of 1.5×10^4 CFU/cavity, and declined with the higher CFU inoculations (2.5×10^4; 1.0×10^5 and 2.0×10^5 CFU/cavity). In the subsequent experiments, the investigated parameters were determined after injection of 1.5×10^4 and 1.0×10^5 CFU/cavity, which were named non-lethal and lethal groups, respectively.

3.2. Effect of compound 48/80 on mast cell depletion, survival rate, bacteremia, neutrophil migration and bacteria in peritoneal cavity

As shown in Fig. 2A, the pretreatment of the mice with compound 48/80 depleted peritoneal mast cells by 84% 24

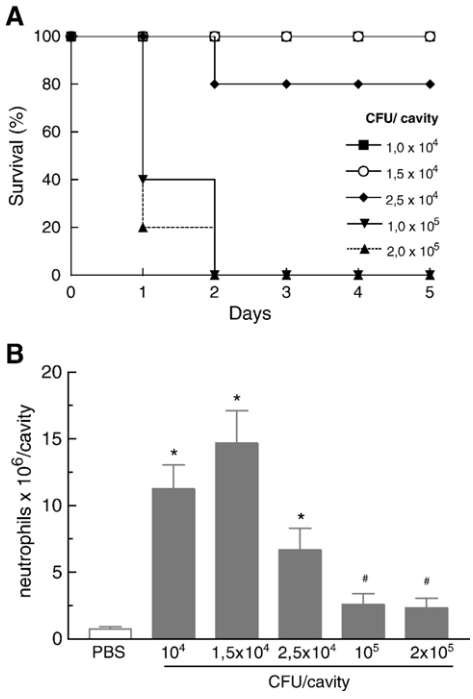

Fig. 1. Survival curve and quantification of neutrophil migration into the peritoneal cavity after bacterial inoculation. Groups of animals received different bacterial inocula (1×10^4, 1.5×10^4, 2.5×10^4, 1×10^5, 2×10^5 CFU/cavity; i.p.). (A) Survival was determined daily until 5 days after bacterial inoculation. Results are expressed as percent survival and are the sum of three different experiments ($P<0.05$, Mantel–Cox log rank test). (B) Neutrophil migration into the peritoneal cavity was quantified 6 h after bacterial inoculation or PBS injection. Results are expressed as mean±S.E.M. neutrophils per cavity ($n=15$). *$P<0.01$ compared with control group (PBS). #$P<0.05$ compared with 1.5×10^4 CFU/cavity (analysis of variance, followed by Bonferroni's test).

h after the last administration of the drug. The mast cell population was restored to 58% of the control level five days after compound 48/80 administration. The compound 48/80 treatment did not affect the macrophage and lymphocyte populations (data not shown in figure).

The lethal group of animals pretreated with compound 48/80 presented 80% survival versus 20% observed in the non-pretreated animals (Fig. 2B) in the first two days after bacterial inoculations. In the last 3 days of observation (3rd–5th days), mice of the group pretreated with compound 48/80 showed 60% survival, whereas the non-treated group showed only 5%. The increase in survival rates of the compound 48/80 group was accompanied by a significant decrease in the number of bacteria in peritoneal exudates and bacteremia (Fig. 2C), compared with the non-pretreated lethal group.

As illustrated in Fig. 2D, pretreatment of the animals with compound 48/80 prevented the neutrophil migration failure observed in the lethal group. Furthermore, the pretreatment with compound 48/80 of non-lethal animals caused a marked increase in neutrophil migration.

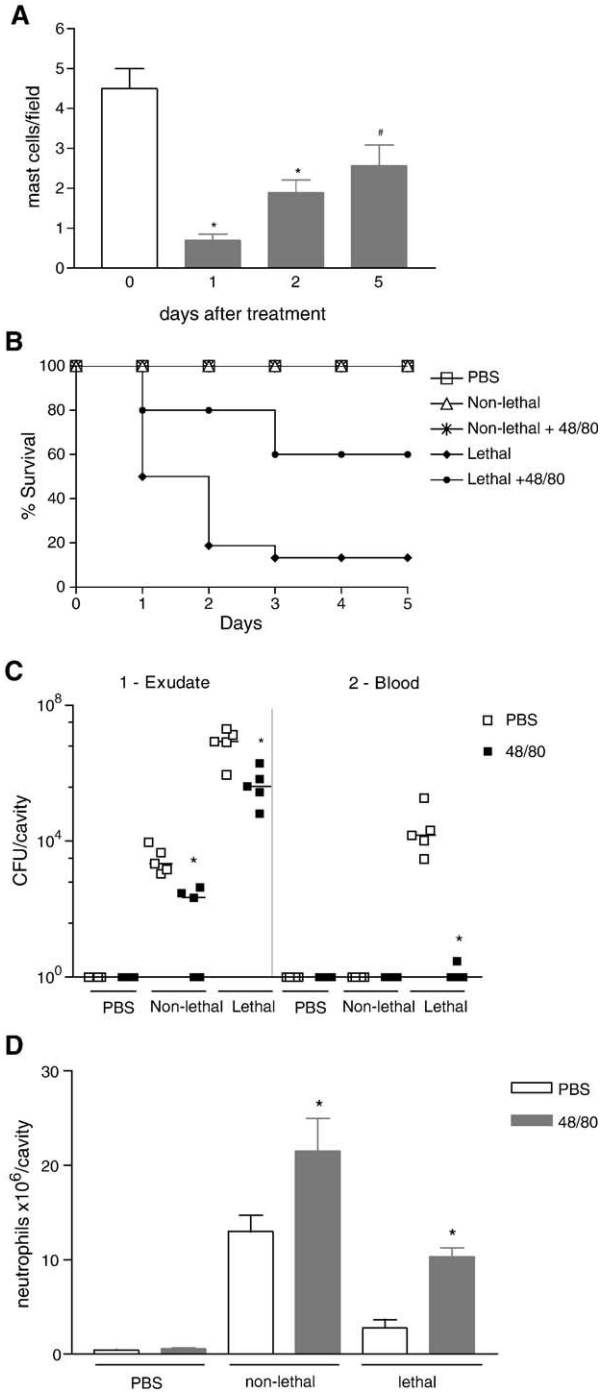

Fig. 2. Effects of compound 48/80 pretreatment on mast cell depletion, survival, bacteria number and neutrophil migration to the peritoneal cavity after bacterial inoculation. Groups of animals were chronically treated with compound 48/80 and received i.p. non-lethal (1.5×10^4 CFU in 300 μl/cavity) or lethal (1.0×10^5 CFU in 300 μl/cavity) bacterial inoculations. A—bars represent the number of mast cells stained by toluidine blue in the peritoneal cavity 1, 2 and 5 days after compound 48/80 treatment (*$P<0.01$ compared with untreated group). B—survival was determined daily until 5 days after bacterial challenge. Results are expressed as % survival and are representative of 3 different experiments ($P<0.05$, Mantel–Cox log rank test). C—the CFU were determined in the exudate and blood collected at 6 h after bacterial inoculum or PBS injection. Results are expressed as CFU per cavity (exudate) or as CFU per ml of blood and are the sum of 2 different experiments. The horizontal bars represent the median of the results. D—the bars show the neutrophil migration into the mouse peritoneal cavity 6 h after bacterial inoculation or PBS injection. Results are expressed as mean±S.E.M. ($n=15$). *$P<0.05$ compared with non-treated animals of the same group (analysis of variance, followed by Bonferroni's test).

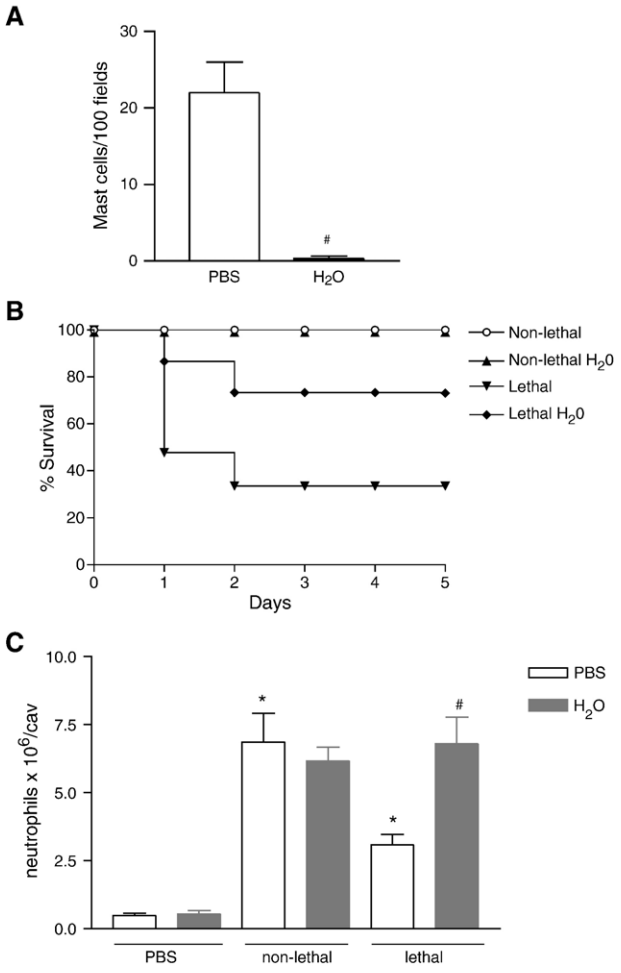

Fig. 3. Effects of distilled water pretreatment on mast cell depletion, survival, bacteria number and neutrophil migration into the peritoneal cavity after bacterial inoculations. Groups of animals were injected with sterile distilled water and received 24 h after i.p. non-lethal (1.5×10^4 CFU/cavity) or lethal (1.0×10^5 CFU/cavity) bacterial inoculations. A—bars show the number of mast cells in the peritoneal cavity 24 h after water injection. B—survival curves were determined daily until 5 days after bacterial inoculations. Results are expressed as % survival and are the sum of 3 different experiments. Survival of lethal group injected with water was significantly different from non-treated lethal mice ($P<0.05$, Mantel–Cox log rank test). C—the bars show the neutrophil migration into the mouse peritoneal cavity 6 h after bacterial inoculation or PBS injection. Results are expressed as mean±S.E.M. ($n=15$). *$P<0.01$ compared with the PBS group and $^{\#}P<0.05$ compared with non-treated animals of lethal group (analysis of variance, followed by Bonferroni's test).

3.3. Effect of distilled water on resident mast cell depletion, survival rate, neutrophil migration and bacteria in the peritoneal cavity

The pretreatment with sterile distilled water also was effective in reducing the number of peritoneal mast cells by more than 95% (Fig. 3A). Distilled water pretreatment did not interfere significantly with the number of resident macrophages and lymphocyte populations (data not shown). The lethal group of animals pretreated with distilled water also presented a significant increase in survival rate (Fig. 3B), and did not exhibit the neutrophil migration failure observed in the nontreated lethal group (Fig. 3C). The pretreatment with distilled water did not alter neutrophil migration to the peritoneal cavity in the non-lethal group (Fig. 3C).

3.4. Serum cytokine and nitrate levels following bacterial inoculation and compound 48/80 pretreatment

Fig. 4 shows the concentrations of TNF-α, IL-1β, IL-10 and NO_3 in the sera, 6 h after bacterial inoculation. The TNF-α and IL-1β levels in the sera increased significantly in the lethal group

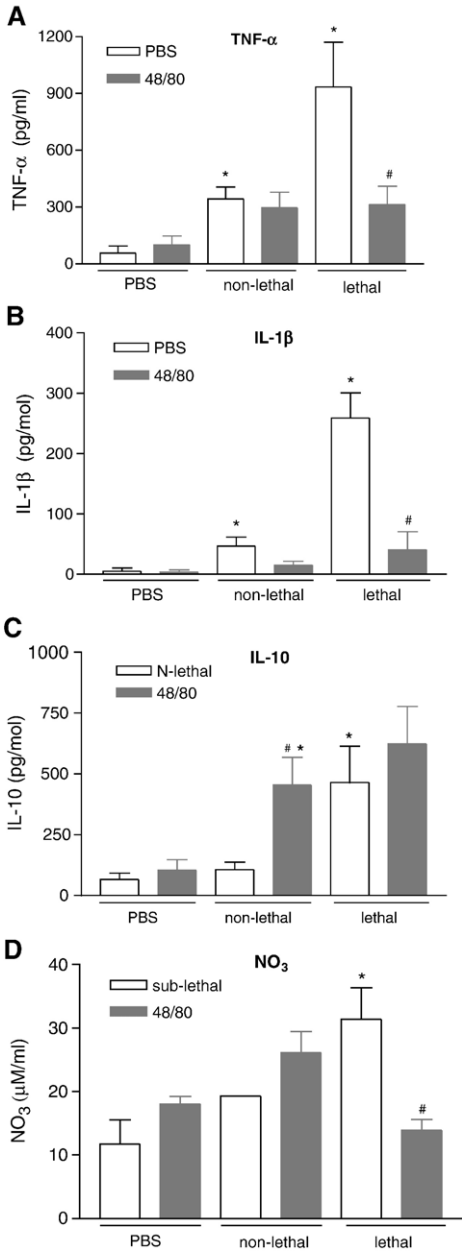

Fig. 4. Serum concentrations of TNF-α, IL-1β, IL-10 and NO_3 of mice injected with PBS, non-lethal (1.5×10^4 CFU/cavity) or lethal (1.0×10^5 CFU/cavity) bacterial inoculations pretreated or not with compound 48/80. The cytokine concentrations in sera were determined 6 h after PBS injection or bacterial inoculations. Results are expressed as mean±S.E.M. of nanograms of cytokine per milliliter of serum and μMolar of NO_3 ($n=7$). *$P<0.05$ compared with PBS i.p. injected mice. $^{\#}P<0.05$ compared with non-treated animals of the same group (analysis of variance, followed by Bonferroni's test).

compared to control and non-lethal group (Fig. 4A and B). The pretreatment with compound 48/80 reduced TNF-α and IL-1β concentrations in sera of the lethal group to levels similar to those observed in the non-lethal group. The serum levels of IL-10 in the lethal group increased significantly compared with non-lethal animals (Fig. 4C). The pretreatment with compound 48/80 increased significantly the IL-10 levels in non-lethal animals but not in the lethal group. Nitrate levels in the animal sera were significantly increased in the lethal group when compared with non-lethal and control animals (Fig. 4D). The compound 48/80 pretreatment significantly blocked the increase of serum NO_3 in lethal animals to levels observed in the PBS-injected group.

3.5. Survival rate, neutrophil migration and bacterial count in air-pouch

Taking into account that air-pouches do not present mast cells as resident cells (19), the non-lethal and lethal doses of bacteria were injected into the air-pouch cavity. No death (Fig. 5A), failure of neutrophil migration (Fig. 5B), or bacteremia (Fig. 5C) were observed when sub-lethal or lethal bacterial inocula were injected in this model. In an attempt to demonstrate that the absence of neutrophil migration failure observed in air-pouches injected with lethal doses of bacteria was a consequence of a lack of systemically released inflammatory mediators, the air-pouches and peritoneal cavities were injected simultaneously with bacterial inocula. These were injected into the peritoneal cavity to induce systemic release of inflammation mediators. When a non-lethal dose of bacteria was injected into the peritoneal cavity, no reduction of neutrophil infiltration into the air-pouch induced by non-lethal (Fig. 6A) or lethal doses of bacteria (Fig. 6B) was observed. However, when a lethal inoculum was injected into the peritoneal cavity, a marked blockade of neutrophil migration into the peritoneal cavity and also to the air-pouch (even when challenged with a non-lethal dose) was observed (Fig. 6C).

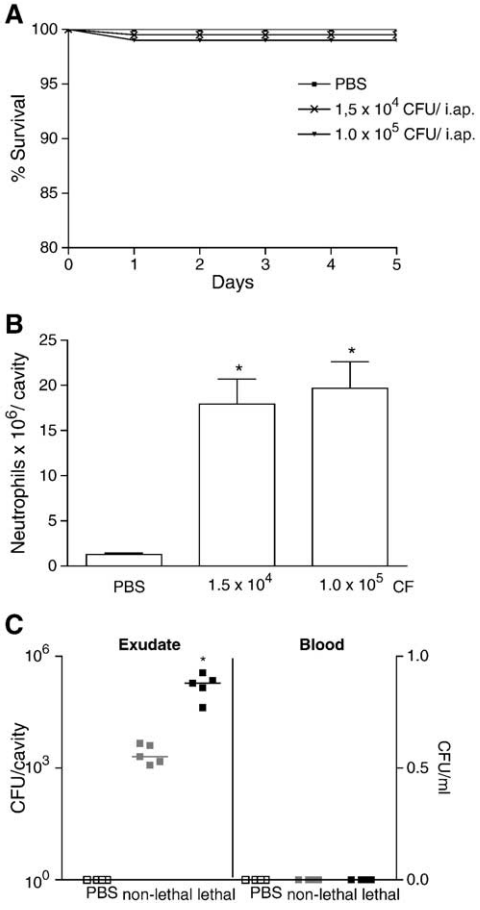

Fig. 5. Survival curves and quantification of neutrophil migration into and CFU in the air-pouches after bacterial inoculations. Air-pouches (i.ap.) were inoculated with 1.5×10^4 CFU (CFU/i.ap.) or 1.0×10^5 CFU. A—survival was determined daily until 5 days after bacterial inoculations. Results are expressed as % survival and are the sum of 3 different experiments. B—neutrophil migration into the air-pouch was quantified 6 h after bacterial inoculation or PBS injection. C—the CFU were determined in the exudate and blood collected 6 h after bacterial inoculum or PBS injection. Results are expressed as CFU per cavity (exudate) or as CFU per ml of blood and are the sum of 2 different experiments ($n=6$). The horizontal bars represent the median of the results. *$P<0.05$ compared to control group (PBS) (analysis of variance, followed by Bonferroni's test).

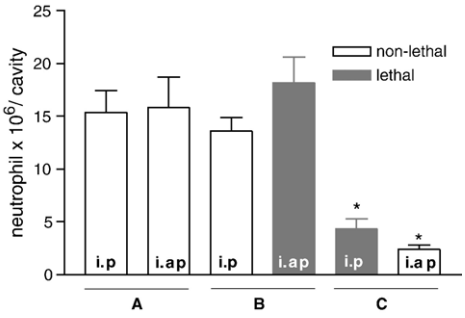

Fig. 6. Neutrophil migration induced by simultaneous bacterial inoculation in the peritoneal and air-pouch cavities. Groups of animals received simultaneously non-lethal (1.5×10^4 CFU/cavity) or lethal (1.0×10^5 CFU/cavity) bacterial inoculations in the peritoneal cavity (i.p) and in the air-pouch (i.ap). The pairs of bars A, B and C indicate neutrophil migration in animals injected simultaneously into peritoneal and air-pouch cavities with 1.5×10^4 CFU/cavity (open bars) or 1.0×10^5 CFU/cavity (grey bars). Neutrophil migration was quantified 6 h after bacterial inoculations. Results are expressed as the mean ±S.E.M. ($n=15$). *$P<0.05$ compared with the same inoculum in panels A or B (analysis of variance, followed by Bonferroni's test).

4. Discussion

In the present study, we investigated the role of mast cells in the pathogenesis of sepsis. It was found that these cells play an important role in the establishment of severe polymicrobial sepsis.

Previous results demonstrated that failure of neutrophil migration to an infectious focus is a key event responsible for the host's inability to restrict the pathogens, allowing their systemic spreading culminating in systemic inflammatory response, septicemic shock and death. Neutrophil migration failure was demonstrated in septicemia induced by *S. aureus*, *E. coli* endotoxin and by cecal ligation and puncture surgery and in septic patients (Crossara-Alberto et al., 2002; Rocha and Ferreira, 1986; Benjamim et al., 2000; Tavares-Murta et al., 2002). In the present study we demonstrated that intraperitoneal administration of lethal polymicrobial inocula obtained from cecum contents induced failure of neutrophil migration to the

infectious focus, which was followed by a high mortality rate, high number of bacteria in the peritoneal cavity and circulation, and high serum concentration of pro-inflammatory cytokines and nitrate (Figs. 1, 2 and 4). The failure of neutrophil migration was not due to neutropenia, because lethal mice have level of circulating neutrophils higher then those observed non-lethal infected mice (data not shown). Thus, despite different models, the severity of sepsis correlates with the degree of neutrophil migration impairment.

In order to verify the participation of mast cells in the neutrophil migration failure, we depleted these cells by pharmacological treatment of the animals with compound 48/80, which leads to impairment of mast cell function through their degranulation (Selvan et al., 1994; Ribeiro et al., 1991); or by administration of distilled water which lyses the mast cells (Medonça et al., 1986). We confirmed that the compound 48/80 treatment significantly reduced the mast cells population of the peritoneal cavity for at least five days. Similarly, the intraperitoneal administration of distilled water depleted the peritoneal resident mast cells by more than 90%. None of the pretreatments had a significant effect on the number of resident mononuclear cells (data not shown).

The failure of neutrophil migration to the infectious focus was not observed when the lethal inoculum of bacteria was administered to peritoneal cavities depleted of mast cells, either by treatment with compound 48/80 or injection of sterile distilled water. Furthermore, failure of neutrophil migration also was not observed when the lethal inocula were injected into the air-pouch cavities, in which mast cells are not present as resident cells (Supajatura et al., 2001). Similarly, there are evidences in the literature that knock-out mice lacking mast cell dipeptidyl peptidase I and histidine decarboxylase are more resistant to cecal ligation and puncture and *E. coli* peritonitis (Mallen-St Clair et al., 2004; Hori et al., 2002).

In apparent contrast with our results, Echtenacher et al. (1996) and Malaviya et al. (1996) observed that mast cell-deficient mice (W/W^v) presented reduced neutrophil migration into the peritoneal cavity with consequent limited clearance of bacteria and high mortality (≈80%) compared to wild type (≈30%) when undergoing cecal ligation and puncture or *Klebsiella pneumoniae* inoculation, respectively. The reduced neutrophil migration to the infectious focus observed in W/W^v in both cases seems to be a consequence of the reduction in TNF-α production, since W/W^v presented reduced levels of this cytokine in the infectious focus. Moreover, TNF-α specific antibodies blocked over 70% of the neutrophil influx into the infectious focus of wild type mice and purified mast cells released TNF-α upon incubation with bacteria. Furthermore, Supajatura et al. (Vassalli, 1992) using W/W^v mice also showed a protective role for mast cells in cecal ligation and puncture-induced sepsis. The use of different experimental models might be the simplest explanation for the apparent contradiction between the deleterious (our study) and protective role of mast cells in sepsis (Echtenacher et al., 1996; Malaviya et al., 1996). It seems that during severe sepsis with the presence of microorganisms and inflammatory mediators in the bloodstream (our study), mast cells contribute to the aggravation of the process with systemic release of more inflammatory mediators, including those involved in the failure of neutrophil migration. In fact, the depletion of mast cells by treatment with compound 48/80, with consequent reestablishment of neutrophil migration reduced the systemic levels of the proinflammatory cytokines TNF-α and IL-1-β. However, during a mild cecal ligation and puncture, as happen in the mentioned studies, mast cells act mainly as a resident cell source of leukocyte chemotactic mediators. Actually, confirming that the infection is mainly confined locally in the mentioned studies, the lethality observed in wild type mice was approximately of only 20%. Furthermore, as mentioned above, the production of TNF-α, a well-known neutrophil chemotactic mediator (Selvan et al., 1994) was reduced by genetic depletion of mast cells, as was the neutrophil migration to the infection site. Moreover, it is well known that mast cells are an important source of chemotactic mediators, such as IL-8, monocyte chemotatic protein 1 (MCP-1), macrophage inflammatory proteins (MIP-1α, MIP-1β) and RANTES (regulated upon activation normal T cell expressed and secreted) (Arguello et al., 1992). However, against this hypothesis, we observed in the present study that mast cell depletion by compound 48/80 pretreatment or water administration did not reduce the neutrophil migration induced by a sub-lethal dose of bacteria that induces mainly a local infection. Moreover, reduction of neutrophil migration was also not observed when a sub-lethal dose of bacteria was injected into the air pouch, a mast cell deficient cavity. These results suggest that other cells are producing the neutrophil chemotactic factor in the infectious focus. It is important to mention that, besides the mast cell deficiency, the W/W^v mice may also exhibit other deficiencies. For instance, the W/W^v mice present pleiotropic defects in pigment-forming cells, germ cells and red blood cells. They also exhibit intrinsic progenitor cell defects that lead to the development of some ovarian tumors associated with an overproduction of pituitary gonadotropic hormone (Murphy, 1972; Ezoe et al., 1995; Tiao et al., 1994). These impairments could interfere with the development of sepsis. Thus, a simultaneous study in mast cell depleted animals by pharmacological (compound 48/80) and genetic (W/W^v) procedures needs to be done to clarify the role of these cells in sub-lethal and lethal sepsis.

Previous studies have shown that the severity of sepsis correlates with systemic release of proinflammatory cytokines and chemokines and a concomitant increase in nitric oxide (NO) derived from inducible NO synthase (iNOS) (Walley et al., 1996; Wolkow, 1998; Court et al., 2002). These mediators are responsible for the most deleterious events observed in sepsis, including cardiovascular changes, multiorgan dysfunction and neutrophil migration failure (Bone et al., 1992; Tavares-Murta et al., 1998; Benjamim et al., 2000). Concerning the neutrophil migration impairment, it was demonstrated that intravenous administration of TNF-α and IL-8 inhibited neutrophil migration induced by different stimuli (Hechtman et al., 1991). Moreover, the impairment of neutrophil migration and reduction of rolling/adhesion found in lethal sepsis induced by cecal ligation and puncture were not observed in iNOS-

deficient mice or in animals treated with aminoguanidine, a selective iNOS inhibitor (Casey et al., 1993). Confirming the deleterious role of mast cells in our model of severe sepsis, we observed that the treatment of the animals with compound 48/80, concomitant with the reestablishment of neutrophil migration with consequent circumscription of the bacteria in the infectious focus and protection from death, reduced systemic concentrations of TNF-α, IL-1β and nitrate. Increases in these cytokines during severe sepsis have been described by others (Oberholzer et al., 2002). The serum concentration of IL-10 was not reduced by the depletion of mast cells, suggesting that it is not involved in the reduction of neutrophil migration, even though it has been described as an anti-inflammatory cytokine and, when produced concomitantly with TNF-α and IL-1β, may counteract the proinflammatory effect of these cytokines (Van Der Meeren et al., 1999; Ahmed et al., 1999).

Reinforcing the idea that during severe sepsis mast cells are an important source of systemic inhibitory mediators of neutrophil migration and that the neutrophil migration impairment is not a phenomenon restricted to the infectious focus, but rather a systemic process, it was observed that when a lethal inoculum of bacteria was injected into the peritoneal cavity, failure of neutrophil migration was observed locally and also into the air pouch. This result is in according to the findings of Ahmed et al. (1999). On the other hand, when the same inoculum was injected into the air-pouch, a cavity poorly populated by mast cells (Supajatura et al., 2001), failure of neutrophil migration was not observed in either cavity.

In conclusion, different from the protective role of mast cells in the innate immunity response to non-severe infections, we demonstrated that these cells exert harmful effects in severe sepsis by mediating the failure of neutrophil migration to the infectious focus through systemic release of inflammatory mediators.

Acknowledgments

CAPES and CNPq for financial support.

We also thank Fabiola Leslie Mestriner, Giuliana Bertozi and Ana Kátia dos Santos for technical assistance.

References

Ahmed, N.A., McGill, S., Yee, J., Hu, F., Michel, R.P., Christou, N.V., 1999. Mechanisms for the diminished neutrophil exsudation to secundary inflammatory sites in infected patients with a systemic inflammatory response (sepsis). Crit. Care Med. 27, 2459–2468.

Arguello, F., Furlanetto, R.W., Baggs, R.B., Graves, B.T., Harwell, S.E., Cohen, H.J., Frantz, C.N., 1992. Incidence and distribution of experimental metastases in mutant mice with defective organ microenvironments (genotypes Sl/Sld and W/Wv). Cancer Res. 52, 2304–2309.

Benjamim, C.F., Ferreira, S.H., Cunha, F.Q., 2000. Role of nitric oxide in the failure of neutrophil migration in sepsis. J. Infect. Dis. 182, 214–223.

Benjamim, C.F., Silva, J.S., Fortes, Z.B., Oliveira, M.A., Ferreira, S.H., Cunha, F.Q., 2002. Inhibition of leukocyte rolling by nitric oxide during sepsis leads to reduced migration of active microbicidal neutrophils. Infect. Immun. 70, 3602–3610.

Bone, R.C., Balk, R.A., Cerra, F.B., Dellinger, R.P., Fein, A.M., Knaus, W.A., Schein, R.M., Sibbald, W.J., 1992. American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. Chest 101, 1644–1655.

Casey, L.C., Balk, R.A., Bone, R.C., 1993. Plasma cytokine and endotoxin levels correlates with survival in patients with the sepsis syndrome. Ann. Intern. Med. 119, 771–778.

Cohen, J., 2002. The immunopathogenesis of sepsis. Nature 420, 885–891.

Court, O., Kumar, A., Parrillo, J.E., 2002. Clinical review: myocardial depression in sepsis and septic shock. Crit. Care Med. 6, 500–508.

Crossara-Alberto, D.P., Darini, A.L.C., Inoue, R.Y., Silva, J.S., Ferreira, S.H., Cunha, F.Q., 2002. Involvement of NO in the failure of neutrophil migration in sepsis induced by *Staphylococcus aureus*. Br. J. Pharmacol. 136, 645–658.

Echtenacher, B., Mannel, D.N., Hultner, L., 1996. Critical protective role of mast cells in a model of acute septic peritonitis. Nature 381, 75–77.

Edwards, J.W.C., Sedgewich, A.D., Willoughby, D.A., 1981. The formation of a structure with the feators of synovial lining by subcutaneous injections of air. An "in vivo" tissue culture system. J. Pathol. 134, 147–156.

Ezoe, K., Holmes, S.A., Ho, L., Bennett, C.P., Bolognia, J.L., Brueton, L., Burn, J., Falabella, R., Gatto, E.M., Ishii, N., 1995. Novel mutations ad deletions of the KIT (steel factor receptor gene in human piebaldism. Am. J. Hum. Genet. 56, 58–66.

Hechtman, D.H., Cybulsky, M.I., Fuchs, H.J., Baker, J.B., Gimbrone Jr., M.A., 1991. Intravascular IL-8. Inhibitor of polymorphonuclear leukocyte accumulation at sites of acute inflammation. J. Immunol. 147, 883–892.

Hirasawa, N., Ohtsu, H., Watanabe, T., Ohuchi, K., 2002. Enhancement of neutrophil infiltration in histidine decarboxylase-deficient mice. Immunology 107, 217–221.

Hori, Y., Nihei, Y., Kurokawa, Y., et al., 2002. Accelerated clearance of *Escherichia coli* in experimental peritonitis of histamine-deficient mice. J. Immunol. 169, 1978–1983.

Malaviya, R., Ikeda, T., Ross, E., Abraham, S.N., 1996. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-α. Nature 381, 77–80.

Mallen-St Clair, J., Pham, C.T., Villalta, S.A., Caughey, G.H., Wolters, P.J., 2004. Mast cell dipeptidyl peptidase I mediates survival from sepsis. J. Clin. Invest. 113, 628–634.

Medonça, V.O., Vugman, I., Jamur, M.C., 1986. Maturation of adult rat peritoneal and mesenteric mast cells. A morphological and histofluorescence study. Cell Tissue Res. 243, 635–639.

Murphy, E.D., 1972. Hyperplastic and early neoplastic changes in the ovaries of mice after genic deletion of germ cells. J. Natl. Cancer Inst. 48, 1283–1295.

Oberholzer, A., Oberholzer, C., Moldawer, L.L., 2002. Interleukin-10: a complex role in the pathogenesis of sepsis syndromes and its potential as an anti-inflammatory drug. Crit. Care Med. 30, 58–63.

Patel, G.P., Gurka, D.P., Balk, R.A., 2003. New treatment strategies for severe sepsis and septic shock. Curr. Opin. Crit. Care 9, 390–396.

Ribeiro, R.A., Flores, C.A., Cunha, F.Q., Ferreira, S.H., 1991. IL-8 causes in vivo neutrophil migration by a cell-dependent mechanism. Immunology 73, 472–477.

Riedemann, N.C., Guo, R., Ward, P.A., 2003. Novel strategies for the treatment of sepsis. Nat. Med. 9, 517–524.

Rocha, N.P, Ferreira, S.H., 1986. Restoration by levamisole of endotoxin-inhibited neutrophil migration, oedema and increased vascular permeability induced by carrageenin. Eur. J. Pharm. 122, 87–92.

Selvan, R.S., Butterfield, J.H., Krangel, M.S., 1994. Expression of multiple chemokine genes by a human mast cell leukemia. J. Biol. Chem. 13 (269), 13893–13898.

Supajatura, V., Ushio, H., Nakao, A., Okumura, K., Ra, C., Ogawa, H., 2001. Protective roles of mast cells against enterobacterial infection are mediated by Toll-like receptor 4. J. Immunol. 15, 2250–2256.

Tavares-Murta, B.M., Cunha, F.Q., Ferreira, S.H., 1998. The intravenous administration of tumor necrosis factor, interleukin-8 and macrophage-derived neutrophil chemotactic factor inhibits neutrophil migration by stimulating nitric oxide production. Brit. J. Pharmacol. 124, 1369–1374.

Tavares-Murta, B.M., Zaparoli, M., Ferreira, R.B., Silva-Vergara, M.L., Oliveira, C.H., Murta, E.F., Ferreira, S.H., Cunha, F.Q., 2002. Failure of neutrophil chemotactic function in septic patients. Crit. Care Med. 30, 1056–1061.

Tiao, G., Rafferty, J., Ogle, C., 1994. Effect of nitric oxide synthase inhibition during endotoxemia may be caused by high levels of tumor necrosis factor and interleukin-6. Surgery 116, 332–338.

Topley, N., Mackenzie, R., Williams, J.D., 1995. Macrophages and mesothelial cells in bacterial peritonitis. Immunobiology 195, 563–573.

Van Der Mereen, A., Squiban, C., Gourmelon, P., Lafont, H., Gaugler, M.H., 1999. Differential regulation by IL-4 and IL-10 of radiation-induced IL-6 and IL-8 production and ICAM-1 expression by human endothelial cells. Cytokine 11, 831–838.

Vassalli, P., 1992. The pathophysiolgy of tumor necrosis factors. Annu. Rev. Immunol. 10, 411–452.

Verdrengh, M., Tarkowiski, A., 1997. Role of neutrophils in experimental septicemia and septic arthritis induced by *Staphylococcus aureus*. Infect. Immun. 65, 2517–2521.

Walley, K.R., Lukacs, N.W., Standiford, T.J., Strieter, R.M., Kunkel, S.L., 1996. Balance of inflammatory cytokines related to severity and mortality of murine sepsis. Infect. Immun. 64, 4733–4738.

Wolkow, P.P., 1998. Involvement and dual effects of nitric oxide in septic shock. Inflamm. Res. 47, 152–166.