

BACKGROUND: Abdominal sepsis induces a local production of proinflammatory mediators that may trigger both septic shock and organ-system dysfunction.

Aims: The present study analyzed exudation, cell migration, and CD11a and CD18 subset cells of both local and systemic responses induced by fecal peritonitis in mice.

Methods: Animals were anesthetized and, after performing a midline incision in the abdomen, the cecum was ligated and punctured twice with a needle. Sham-operated animals were included. Some groups were previously treated with Evans blue dye (intravenously) to further evaluate the amount of tissue and abdominal cavity leakages.

Results: Fecal peritonitis triggered a local inflammatory reaction with an increased number of leukocytes and exudation between 6 and 48 h ($p < 0.01$). Although CD11a/CD18-positive cells in the abdomen peaked after 24 h, a significant decrease of them was detected after 48 h ($p < 0.05$). At the studied period of time (6–48 h), different degrees of exudation in several organs occurred, whereas a significant late recruitment (24 h) of CD11a/CD18 cells into the lungs was observed.

Conclusions: In this model, cell migration and exudation at the site of injury occurred in parallel. However, in the lungs, the recruitment of leukocytes that express CD11a/CD18 adhesion molecules constitutes a non-dependent event in relation to fluid leakage accumulation at this site.

Key words: Polymicrobial peritonitis, Sepsis, CD11a/CD18 subsets, Leukocytes, Exudation, Systemic inflammation, Organs

Analysis of local and systemic inflammatory responses induced by polymicrobial peritonitis in mice

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Introduction

Abdominal sepsis is a leading cause of septic shock and organ-system dysfunction.^{1,2} This severe inflammatory response results from the fact that, regardless of the nature of the infection, the local production of proinflammatory mediators exerts complex and interdependent effects on several organs and in the vasculature. In recent years, the identification of specific molecules that mediated cell adhesion opened a new era in the understanding of this syndrome.^{3,4} In this regard, animal and clinical studies have demonstrated that the adhesion glycoprotein complex CD11a/CD18 is important in mediating neutrophil accumulation at sites of inflammation in many experimental models.⁵ These adhesion molecules are integrins that exist in resting conditions in a state of low affinity for their ligands. At the inflammatory site, appropriated stimuli induced by either proinflammatory cytokines or nuclear factor-kappa B can convert these adhesion molecules, which are

present in leukocyte and endothelium cells, to a high affinity state for their ligands.⁶ This event is followed by a cascade of metabolic steps that provide firm attachment of the neutrophil and permit its migration towards the source of chemoattractants.^{2,7–9} The importance of the role of these adhesion molecules has recently been supported by studies with either transgenic⁴ or knockout mice for these leukocyte adhesion molecules. Further studies have demonstrated, however, that the recruitment of neutrophils to the sites of inflammation can be either CD11a/CD18-dependent or CD11a/CD18-non-dependent according to the organ and to the stimulus used to induce the inflammatory process.^{10,11}

Several experimental models of sepsis are currently employed to examine the different aspects of both local and systemic body responses to this condition.^{12–15} Among them, the mouse model of cecum ligation and puncture induces spillage of the intestinal flora into the peritoneal cavity, leading to diaphragmatic lymphatic clearance and systemic bacteremia

and endotoxemia.¹⁶ Considering that this model provides a suitable approach to evaluate the severity and temporal manifestations induced by sepsis, the aim of this work was: (1) to investigate the kinetics of leukocyte and fluid changes induced by fecal peritonitis either in the peripheral blood or in the abdominal cavity of mice; and (2) to evaluate the systemic inflammatory response induced by polymicrobial peritonitis by measuring fluid leakage as a marker of exudation in several organs. In addition, we tested the hypothesis that the levels of positive CD11a/CD18 adhesion molecules in the leukocytes from blood, abdominal cavity, lungs and spleen significantly varied along the time.

Methods

Animals

Non-fasted adult Swiss mice of both sexes (25–30 g) aged 2 months were used. The animals were maintained in an environment of controlled temperature ($21 \pm 2^\circ\text{C}$), and light–dark cycles of 12 h each, with free access to food and water. Throughout the experiments, the animals were managed using the principles and guidelines for the care of laboratory animals according to Zimmermann.¹⁷

Experimental design

Model of experimental sepsis

The animals were anesthetized with ether inhalation, and a 2 cm ventral midline incision was performed. The cecum was then exposed, ligated just distally to the ileocecal valve, punctured twice with an 18-gauge needle, and returned to the abdominal cavity. The abdominal incision was then closed in two layers. Control animals were sham-operated and underwent the same surgical procedure without cecal ligation and puncture. Following surgery, animals were returned to their respective cages and allowed free access to food and water until sacrifice.

In a first set of experiments, control and septic animals were maintained in an appropriated environment as already described to evaluate the survival rate (Fig. 1). As shown, the average survival rate of septic groups was 5 days, whereas no death was observed in the control group. Based on the survival rate, different periods of time (6, 10, 24 and 48 h) were chosen to analyze the studied parameters described later.

Determination of cell migration and fluid leakage

The amount of exudation in the abdomen and studied organs (liver, spleen, heart, lung and kidney) and the amount of cell migration (total and differential leukocytes $\times 10^6$) in the abdomen were determined at prior-chosen periods of time (6, 10, 24 and 48 h) in both groups (control and septic). To evaluate the

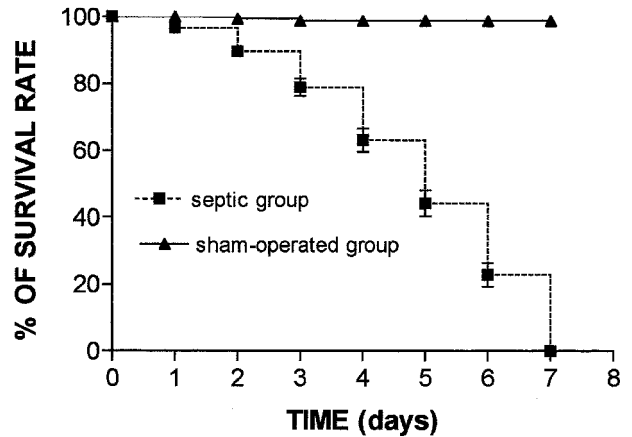


FIG. 1. Median survival rates of the septic mouse group. Each symbol represents the median of at least 10 animals.

degree of fluid leakage, the animals were treated with Evans blue dye (25 mg/kg, intravenously), 1 h before either the induction of peritonitis or the sham-operation procedure. In this set of experiments, samples of blood obtained by cardiac puncture were also harvested to analyze blood cells.

At the established periods of time, animals were killed with an overdose of ether. Following death, the abdominal cavity was washed with 1 ml of phosphate-buffered saline (PBS) plus heparin (20 IU/ml), and aliquots (500 μl) of the lavage fluid were obtained. At this time, the studied organs were also removed and cleared from both adjacent tissues and blood in order to assess fluid leakage.

Total leukocyte counts and the hematocrit values from either whole blood or peritoneal lavage fluid were determined with an automatic counter machine (Coulter, USA). In addition, cytospin preparations of these samples were stained with May–Grunwald–Giemsa for differential cell counting (mononuclear and polymorphonuclear cells) by microscopy with an oil immersion objective.

The amount of dye in the samples of the peritoneal lavage fluid was measured using colorimetry (Compu-Espectro Spectrometer, Brazil) at 600 nm by interpolation from a standard curve of Evans blue dye in the range 0.01–50 $\mu\text{g/ml}$. Regarding the determination of fluid leakage in the studied organs, after individual determination of each respective wet weight, each tissue was trimmed and incubated with 2 ml of formamide at 45°C for 72 h to extract the Evans blue. The dye was then quantified as previously described and the results expressed as micrograms per gram of wet tissue.

Flow cytometry analysis in the leukocytes from blood, abdominal cavity and studied organs

A pool ($n = 3\text{--}4$ animals from each experimental group) of blood (4.5 nM of ethylenediaminetetraacetic acid (EDTA)-coated tubes) and of fluid leakage sam-

ples from the peritoneal cavity (pre-chilled polypropylene test tubes) were collected at different periods of time after either peritonitis or sham-operation procedures. Samples from the pooled peritoneal lavage were centrifuged ($50 \times g$ for 5 min) and the pellets washed twice in PBS plus azide 1% solution (1 ml). To prevent non-specific binding, samples were treated with 50 μ l of goat serum (pre-treated at 56°C for 30 min, 1:10 dilution in PBS). In other experiments, lymphocytes were isolated from either lungs or spleen ($n = 3-4$ animals from each experimental group) on Ficoll-Hypaque (density 1077) gradients. The mononuclear layer was then removed, washed with PBS (500 μ l) and resuspended again with lysing solution (Becton and Dickinson, USA).

Afterwards, samples of 10^6 cells (50 μ l) were labeled with the appropriated concentration of isothiocyanate of fluorescein (FITC)-conjugated CD11a, CD18 and CD45 mouse antibodies (Sigma Chemical CO, St Louis, MO, USA), for 30 min at room temperature, in the dark. Non-specific staining was controlled by incubation of the cells with their respective isotype control antibodies. After this, the samples were treated with lysing solution (500 μ l) (whole blood (Coulter), peritoneal washes and lymphocytes (Becton and Dickinson)). Finally, analysis was performed on a FACSCalibur flow cytometer (Becton and Dickinson). Gates were set on the different types of cells with the use of forward and side scatters, and a fluorescence intensity of 5000 events was recorded as the mean channel number over a log scale of increasing fluorescence intensity. After determining the percentage of each cell marker subset in both blood and abdominal aliquots, the respective absolute total and differential cell numbers were calculated based on the absolute counts obtained with an automated leukocyte differential system (Coulter).

Drugs

Ficoll-Hypaque 1077 (Inlab, Brazil), FITC-conjugated monoclonal antibodies to CD45, CD11a and CD18, sodium azide (Sigma Chemical Co.), lysing buffer solution (Coulter; Becton and Dickinson), Evans blue dye and EDTA (Merck, Brazil), sterile saline solution (0.9%), PBS (pH 7.6; composition: NaCl, 137 mmol; KCl, 2.7 mmol; phosphate buffer salts, 10 mmol) from different commercial sources were used.

Statistical analysis

The Kaplan-Meier estimate was used to calculate the median survival rates, and the log-rank statistic was used to compare survival values among control and septic groups. Unless otherwise indicated, data are reported as mean \pm SEM. Statistical differences between groups were determined by analysis of variance, complemented with Dunnett's test or by

Student's unpaired "t" test when indicated, and $p < 0.05$ was considered indicative of significance.

Results

After 6 h of peritonitis induction, a marked decrease in the blood leukocyte levels was detected in comparison with the values of the control group (Fig. 2A; $p < 0.01$), being followed by variable levels of increased leukocyte number in the next 48 h period of observation. By contrast, cell migration into the peritoneal cavity was significantly increased at 6 h but remained unchanged up to 10 h, then rose to a maximum between 24 and 48 h ($p < 0.01$). It is noteworthy that, throughout this period of observation, no changes in the hematocrit levels were detected in the septic group, indicating absence of hemorrhagic phenomena (results not shown).

Changes in the leukocyte content of the peritoneal cavity were due to modifications in the neutrophil levels in the intervals 6-8 h after peritonitis induction, whereas in the same experimental conditions both neutrophil and mononuclear cells significantly increased between 10 and 48 h (Fig. 2B). In addition, a significant increase in fluid leakage in the abdominal cavity was detected 4 h after peritonitis induction, and it peaked between 6 and 24 h ($p < 0.01$) (Fig. 2B, inset).

Figure 3 shows the time-course profile of the percentual variation of fluid leakage in the organs obtained from septic animals in comparison with the values obtained from the control group. As shown, this parameter varied greatly both with time and in relation to the tissue studied. In comparison with the control group, the maximal increase of fluid leakage in the kidneys and lungs occurred 10 h after peritonitis induction, whereas in the heart and liver it was detected at 24 h. At 48 h, in all studied organs, except the spleen, no significant accumulation of fluid was detected ($p > 0.05$).

Regarding the percentage of positive cells for CD11a and CD18 in the blood leukocytes from septic animals, changes in the values of each marker did not match in parallel. As presented in Table 1, a significant enhancement of both markers in the abdominal cavity occurred 24 h after peritonitis. At the same time, a marked decay of CD11a, but not of CD18, levels occurred in the blood. In relation to CD18 levels in the blood leukocytes, a significant decrease in these cells was detected at 6 h, but not at 24 h. However, 48 h after peritonitis induction, the levels of both markers either in the blood or in the abdominal cavity was significantly decreased. The source of positive CD11a/CD18 leukocytes was from both mononuclear and polymorphonuclear cells.

Flow cytometry analysis of lymphocytes from lungs indicated a significant recruitment of CD11a/CD18-positive lymphocytes, 48 h after peritonitis

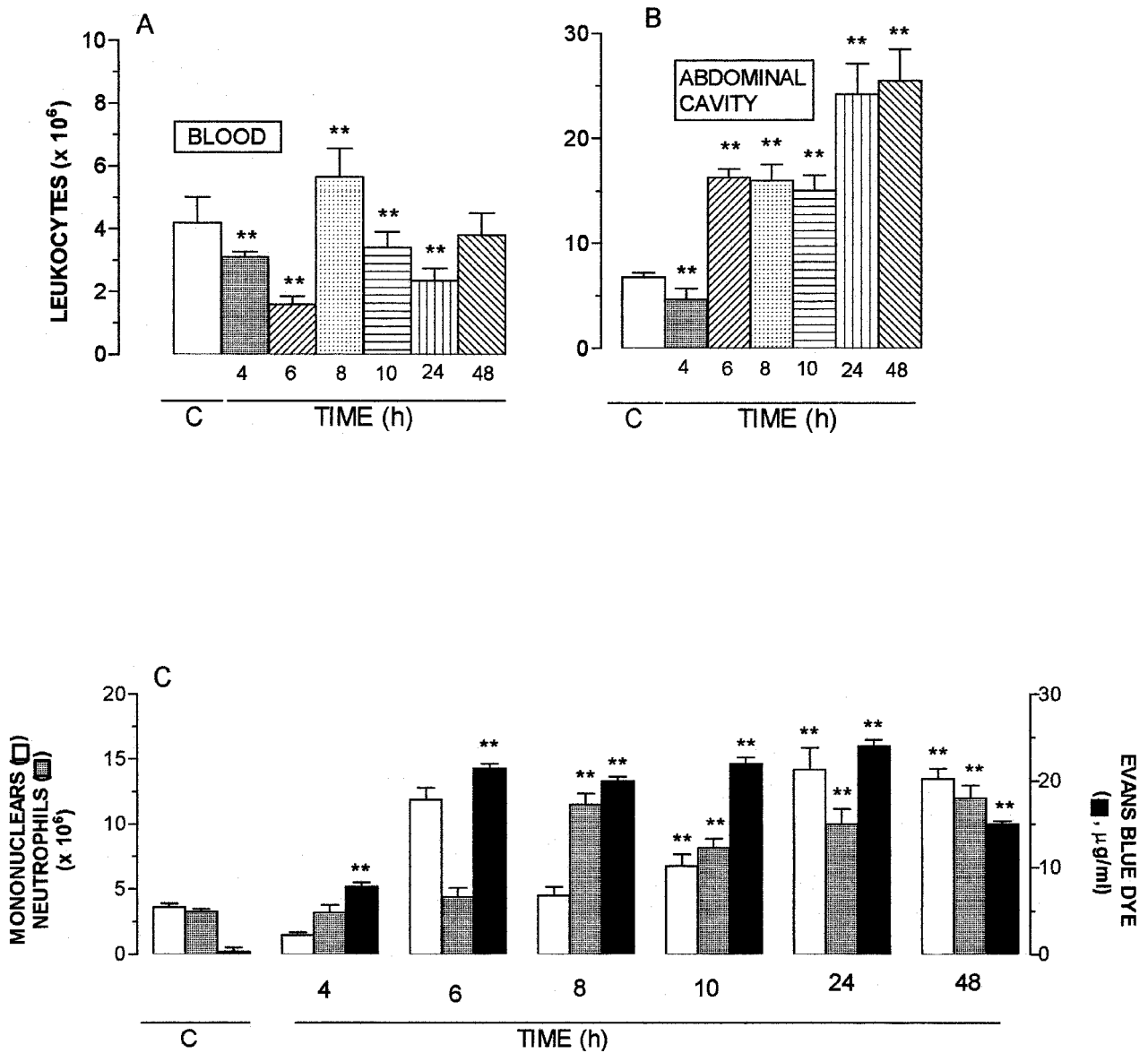


FIG. 2. Kinetics of leukocyte changes in the blood (A) and in the abdominal cavity (B) induced by polymicrobial peritonitis. Number of mononuclear, neutrophil cells and Evans blue leakage (C) in the abdominal cavity. (C) Control (sham-operated animals). Each column represents the mean of a pool of four to six animals, and the vertical bars the SEM. ** $p < 0.01$.

induction, in comparison with the control group. This enhancement, however, was more evident in relation to the CD18 marker. In the spleen, an increase in the percentage of CD11a and CD18 lymphocytes was detected only at 24 h after peritonitis induction (Fig. 4).

Discussion

Our results show that, in this model of non-reversible sepsis, the profile of both leukocyte and exudation amounts at the different sites of study varied greatly. In the first 6 h after peritonitis induction, there was an increased leukocyte migration to the abdominal cavity associated with a sharp decline of leukocyte levels in

the blood. In the next 48 h, the amounts of leukocytes in both compartments (blood and abdominal cavity) were elevated. However, throughout this period of observation, the levels of CD11a/CD18 subset cells varied greatly, indicating that striking differences in the function of the studied leukocytes may exist with time.

Considering that differences in tissue perfusion occur with time in sepsis, cecal ligation associated with punctures provides a suitable model for the analysis of the kinetic profiles of cells and leakage either in the peripheral blood, abdominal cavity, or in other organs. Thus, in parallel to cell migration into the abdominal cavity 6 h following peritonitis induction, an enhancement of fluid accumulation was

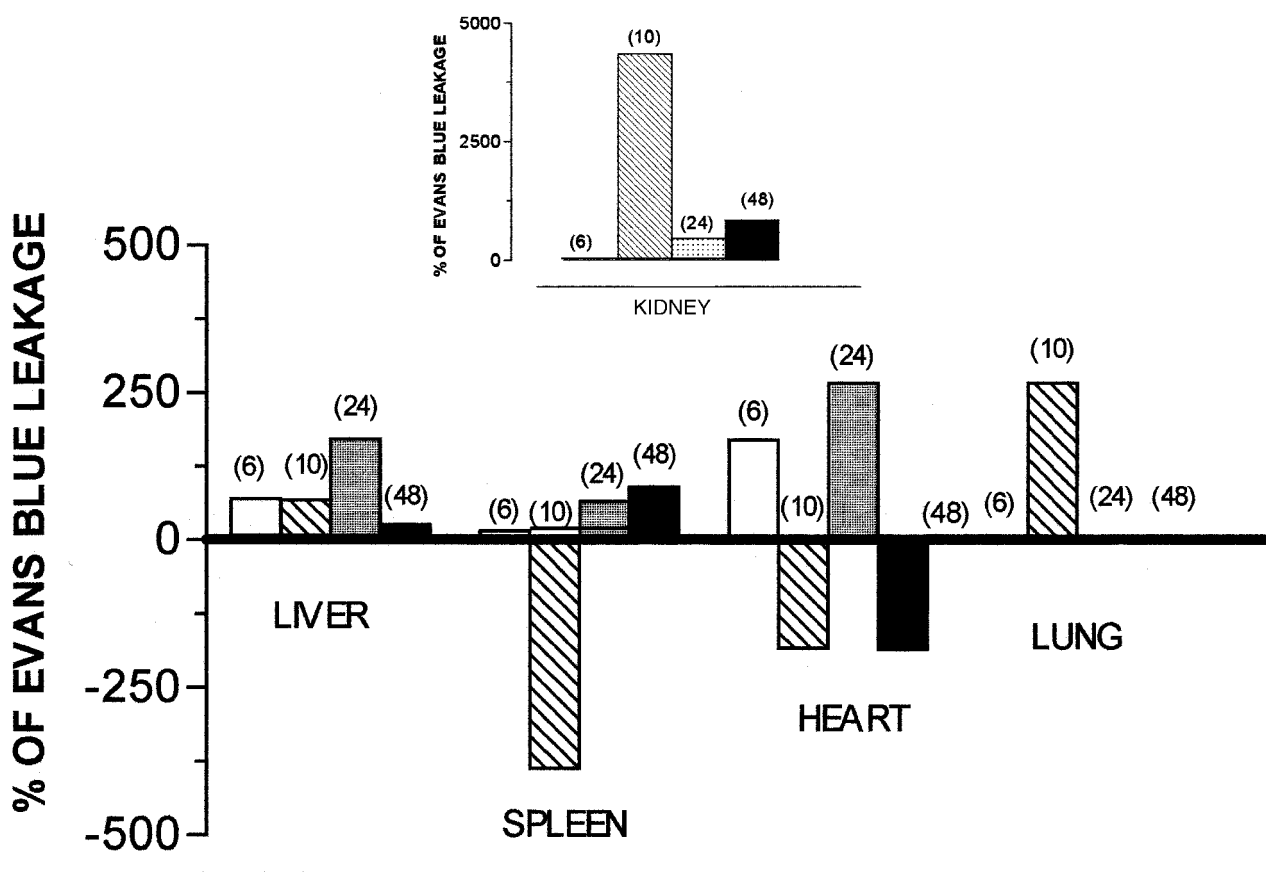


FIG. 3. Time-course profile of fluid leakage in several organs after peritonitis induction. Values are expressed as a percentage in comparison with controls (sham-operated groups). Inset: Same changes in the kidney. Numbers in parentheses indicate the time (h) of evaluation after peritonitis induction. Each column represents the mean values of a pool of four to six animals.

Table 1. Changes in CD11a and CD18 subset cells in the leukocytes harvested from blood and from the abdominal cavity in the studied groups

Groups	Blood ($\times 10^6$)	Abdominal cavity ($\times 10^6$)	Blood		Abdominal cavity	
			CD11a	CD18	CD11a (% of positive cells)	CD18 (% of positive cells)
Total cell						
Control	4.2 \pm 0.2	6.8 \pm 0.4	74.0	83.0	38.0	54.4
6 h	1.6 \pm 0.2	16.3 \pm 0.8	47.5	40.0	36.0	56.0
24 h	2.3 \pm 0.4	24.2 \pm 2.9	12.5	76.0	64.7	84.0
48 h	3.8 \pm 0.7	25.5 \pm 5.4	15.5	9.0	7.0	6.5
Neutrophils						
Control	2.0 \pm 0.2	3.3 \pm 0.2	4.0	18.6	4.8	47.5
6 h	1.2 \pm 0.1	4.4 \pm 0.7	0.4	6.0	12.5	27.5
24 h	1.9 \pm 0.5	10.0 \pm 1.2	0.0	4.0	41.6	55.0
48 h	2.2 \pm 1.6	12.0 \pm 1.0	2.1	1.0	0.0	51.0
Mononuclears						
Control	2.2 \pm 0.5	3.6 \pm 0.3	50.0	60.0	20.0	50.0
6 h	0.5 \pm 0.3	11.9 \pm 0.9	3.0	96.0	35.0	55.0
24 h	0.5 \pm 0.1	14.2 \pm 1.7	0.5	75.0	60.0	80.0
48 h	1.6 \pm 0.5	13.5 \pm 0.8	0.2	10.0	80.0	92.0

Control, Results obtained in sham-operated mice. Each value represents the mean \pm SEM obtained from a pool of three or four animals for each set of experiments.

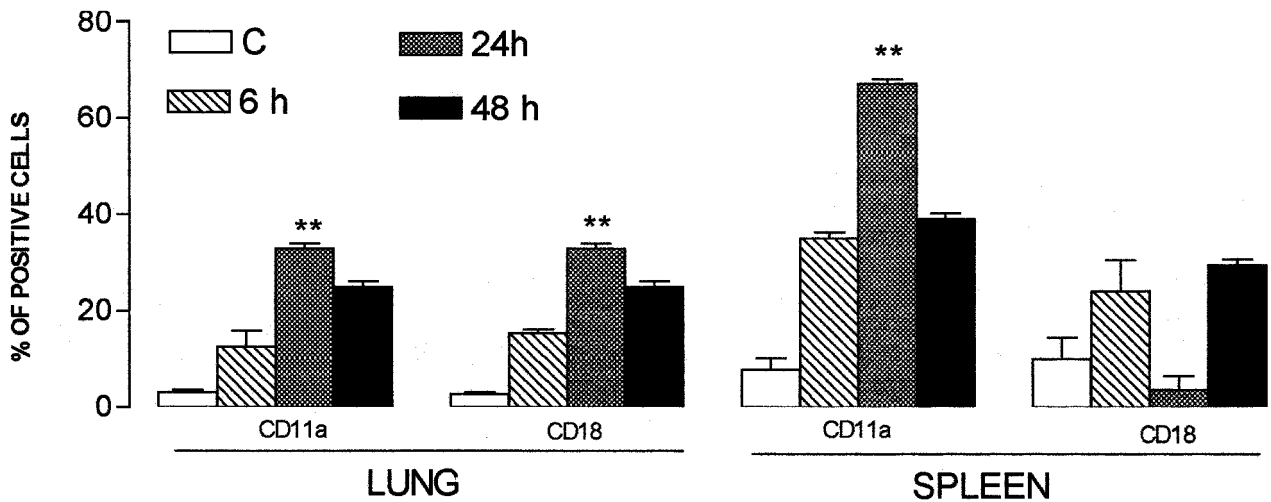


FIG. 4. Time-course profile of the percentage of positive CD11a and CD18 lymphocytes in the lung and spleen obtained from control (C) and peritonitis-induced groups. Each column represents the mean of a pool of two to four organs from at least four to six animals in each experimental group. The vertical bars indicate the SEM. ** $p < 0.01$.

already detected in both the liver and the heart. These findings, however, were also not uniform with time, since at 10 h the opposite effect was observed in the spleen, whereas it remained unchanged in the liver. At this time, maximal fluid accumulation occurred in both lungs and kidneys, but decreased dramatically at 24 and 48 h. Altogether, these variations may be reflecting the different patterns of tissue perfusion due to the circulatory failure that is being established in this non-reversible condition.^{18,19} These results are supported by other studies^{20,21} where it has been shown that the events responsible for the transition from hyperdynamic to hypodynamic sepsis phases are, in part, mediated by the non-uniform generation of nitric oxide by the endothelial cells. Otherwise, the possibility that the temporal profile of the systemic inflammatory response due to the abdominal injury varied as a function of the magnitude of the injury, the importance of the organ and the type of tissue also cannot be discarded.

Regarding cell migration, several studies have demonstrated that adequate leukocyte emigration from the blood cells into the injured tissues is dependent on both CD11a/CD18 adhesion molecules.^{3,10,22,23} In relation to our study, analysis of our data shows that, 6 h after peritonitis induction, the marked fall of blood leukocytes was also associated with a decrease in the percentage of CD11a/CD18 cell subsets. In the next 24 h, despite the fact that the levels of blood leukocytes were elevated, the percentage of CD11a-positive leukocytes further decreased, whereas the percentage of CD18-positive cells was restored. By contrast, in the abdominal cavity, marked recruitment of leukocytes that express CD11a/CD18 was detected only at 24 h later. Taken together, these findings suggest that cell influx 6 h after peritonitis induction is already activated, and a

possible explanation for the low levels of CD11a in the leukocytes from the blood or from the abdominal cavity is that they reflect adherence of these cells on either the endothelium or in the serosa cells of the abdominal cavity due to the upregulation of these cell adhesion ligands. Moreover, it is also concluded that changes in both adhesion molecules are not closely linked, indicating that they may exert different functions in the process of cell recruitment to the site of inflammation. Another interesting aspect of our work was the finding that although leukocyte migration into the abdominal cavity was still detected 48 h after peritonitis induction, the same was not observed in relation to the presence of CD11a/CD18 adhesion molecules. At this time, the percentage values of these integrins were the lowest throughout the studied periods of time. If we consider that the median survival rate of the animals with peritonitis was 5 days, these data altogether point to an early failure in the maintenance of the proinflammatory mechanisms triggered by the body against infection. Another alternative explanation for these findings may be based on the idea that these adhesion molecules, which are essential to cell migration guidance, are no longer necessary once they have achieved their goal.

Analysis of the presented data also provides additional evidence that the systemic inflammatory response induced by polymicrobial peritonitis is highly variable. This conclusion is based on the fact that, in both lungs and spleen, an increased recruitment of leukocytes that express CD11a/CD18 adhesion molecules occurred 24 h after peritonitis induction, a finding that was completely dissociated from fluid leakage volume in both organs. Furthermore, enhanced levels of positive CD11a/CD18 adhesion molecules still persist in the lung after 48 h, indicating

the presence of a systemic inflammatory response to peritonitis. In this context, after induction of experimental fecal peritonitis, sustained increases in the expression of both tumor necrosis factor- α and interleukin-1 β messenger RNA in the lung has also been shown.²⁴⁻²⁶ Altogether, these findings suggest that the local production of cytokines may upregulate the affinity state of these adhesion molecules. Thus, this event certainly facilitates cell migration into the lungs, which in turn contributes to the respiratory failure that occurs in severe septic conditions.

In summary, our results provide additional evidence that the temporal manifestations of the local inflammatory reaction induced by peritonitis triggers a systemic inflammatory response that shows different degrees of fluid accumulation in several organs with time. In addition, the recruitment of leukocytes that express CD11a/CD18 adhesion molecules into the abdominal cavity constitutes a non-dependent event in relation to both cell migration and fluid leakage. It is also suggested that the increased expression in the lungs of these adhesion molecules in the leukocytes may constitute early evidence of the degree of severity of this systemic inflammatory response.

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