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DISSEMINATION OF BACTERIA IN MULTIPLE ORGANS ASSOCIATED WITH APOPTOSIS AND MACROPHAGE ACTIVITY IN DIFFERENT STAGES OF EXPERIMENTAL SEPSIS

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ABSTRACT

Background: **Gram negative sepsis is reported to induce massive translocation of bacteria into tissues, which associates with decreased macrophage function and increased macrophage apoptosis.**

Aims: **The objective of this study was to detect the translocation of bacteria into different organs and to evaluate macrophage activity and the apoptosis of macrophages in the liver during different stages of sepsis and to correlate these parameters.**

Material: **Wistar rats (n = 43) were inoculated intraperitoneally with an E. coli and divided into 5 groups, which were killed at different times.**

Methods: **Counts of translocated bacteria in tissues were evaluated by using morphological and bacteriological methods. Macrophage activity and apoptotic cells in the liver were studied by applying immunohistochemical methods.**

Results: **The counts of E. coli were the highest in the organs and blood 6 h after the onset of sepsis, being in correlation with the highest counts of apoptotic cells in the liver and the falling counts of activated macrophages. The counts of microbes show a new wave of elevation in tissues by 120th h.**

Conclusions: **The massive penetration of bacteria, the depressed macrophage response in early sepsis following the increased rate of apoptotic macrophages, the different rate of bacterial multiplication in tissues and blood and the second wave of the multiplication of bacteria in tissues in late sepsis all refer to the significance of developing immune dysfunction.**

Key words: Sepsis; E. coli; microfoci; apoptosis; macrophages

INTRODUCTION

The general concept of severe sepsis and the septic shock that exists today is a complex sequence of pathophysiological, biochemical and pathomorphological changes leading to death (1, 2, 3). Although bacterial translocation is clearly understood, the causative factors and mechanisms that cause mortality still remain unclear. Numerous current experimental studies have suggested that severe sepsis induces a marked suppression in both the lymphocytic and macrophage function (4, 5), playing a central role in orchestrating the immune response to foreign pathogens (6, 7). Moreover, the depressed macrophage response could be associated with an increased rate of apoptotic macrophage cells in tissues,

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TABLE 1

Results of histological examination of tissue samples (contamination index – CI) of different groups 2, 3, 4 and 5.

	Group II; 6 h						Group III; 24 h						Group IV; 48 h						Group V; 5 day						**Mean**
Lung	2	1	2	1	3	3	0	1	1	0	2	3	1	2	0	1	1	1	1	3	0	0	2	0	**1**
Heart	1	1	1	0	1	1	1	1	0	0	2	1	1	2	1	2	0	1	2	2	2	0	2	0	**0,8**
Spleen	3	1	3	3	2	3	2	1	1	1	1	1	1	1	0	3	0	0	1	na	1	0	1	0	**1,1**
Pancreas	2	1	1	1	na	1	1	na	0	0	2	2	0	0	0	0	0	0	0	na	3	0	0	0	**0,5**
Liver	3	3	2	2	1	3	3	2	1	0	2	1	1	1	0	1	1	1	1	1	1	0	3	0	**1,2**
Adrenals	na	2	0	2	1	2	0	1	0	0	0	1	na	2	0	0	0	0	1	2	1	0	0	0	**0,5**
Kidney	3	1	2	na	2	3	1	2	1	1	3	1	1	2	0	2	0	1	1	3	3	0	2	0	**1,2**
Brain	2	0	0	0	0	0	0	0	0	0	0	1	3	1	1	1	1	1	0	2	1	0	1	0	**0,5**
Ileum	2	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	0	1	na	0	1	0	0	**0,8**
Caecum	1	na	1	1	1	1	na	1	1	0	1	1	1	1	1	na	0	1	1	1	na	1	0	1	**0,8**
Muscle	1	2	1	1	na	na	2	3	0	0	1	1	0	2	0	0	0	1	0	3	na	0	2	0	**0,7**
Stomach	1	1	1	1	1	0	1	1	2	0	1	1	1	2	1	2	1	na	1	2	1	1	1	0	**0,9**
Mean	**1,9**	**1,3**	**1,3**	**1,2**	**1,3**	**1,5**	**1**	**1,3**	**0,7**	**0,3**	**1,3**	**1,3**	**1**	**1,4**	**0,4**	**1,2**	**0,4**	**0,6**	**0,8**	**2,1**	**1,3**	**0,3**	**1,1**	**0,1**	

CI contamination index; na – not available.

being an important mechanism of cell death in sepsis (8). A great deal of attention has been given to the biochemical mechanisms – humoral and cellular immunity and much less to the role of these mechanisms in correlation with the translocation of bacteria in different stages of sepsis (9, 10). The present experimental work has been evolved from our earlier clinical work concerning the septic shock with disseminated microfoci in multiple organs in humans done in the Intensive Care Unit of the Department of Anesthesiology and Intensive Care, University of Tartu. A number of questions were induced by the analysis of the patients who died of the septic shock despite adequate antibacterial therapy, and supportive measurements were assessed in protection against translocation (11, 12). The aim of the study was to set up an experimental model of Gram-negative sepsis in rats, to detect the translocation of bacteria into different organs, to evaluate macrophage activity and a programmed cell death in the rats' liver during different stages of sepsis and to determine the correlation between these parameters.

MATERIAL AND METHODS

Experimental animals – Wistar rats (n = 43, median weight 241 g) were inoculated intraperitoneally with an E. coli, isolated from the blood of septic patients. Viable E. coli cells (2×10^7 g/of body weight) were suspended in 1.5 ml of saline and inoculated intraperitoneally. Final dose used was received after several experiments with different E. Coli doses starting with a 3 log lower dose. Isolated bacteria were identified by using common tests (e.g. Kligler agar, SIM agar and the indole test for E. coli). Thirteen rats died during the experiment and were excluded from the analysis. The surviving 30 rats were killed by cervical dislocation, after which the abdomen and the thorax were scrubbed and shaved with 70 % ethanol. To avoid contamination from the environment and indigenous microflora we used common aseptic techniques (skin and peritoneum disinfection etc.). All organ samples of control animals were sterile. To minimize the possibility of contamination from the infected peritoneal cavity several precautions were used: heart blood was taken, the brain, the muscles and the lungs were removed before the opening of the peritoneal cavity; intraperitoneal organs were washed several times (5×10 ml) in saline before homogenisation and cultivation. Surface cultures of intraperitoneal organs were prepared during our previous studies to ensure the effectiveness of these measures. The animals were killed at different times after inoculation – 2 hours (I Group – 6 rats), 6 h (II Group – 6 rats), 24 h (III Group – 6 rats), 48 h (IV Group – 6 rats), 5 days (V Group – 6 rats). 7 rats serving as control animals and inoculated with 1.5 ml of saline intraperitoneally remained asymptomatic.

HISTOLOGICAL METHOD

Autopsy of the killed animals was performed immediately, samples for histological investigation were randomly taken from 12 organs – the lungs, heart, spleen, pancreas, liver, adrenals, kidney, stomach, small and large intestine, sceletal muscles and the brain of all the animals. For histological evaluation the tissue samples were fixed in 10 % buffered formalin and embedded in paraffin. Sections of 7 µ were cut. Staining of bacteria was carried out according to the method of Gram, modified by Brown and Hopps (13). All the investigations were carried out with the microscope AxioPhot 2 by light (ocul. 10×, obj. 100×). The amount of bacteria was estimated semiquantitatively, whereas a number score, the contamination index (CI) was applied (Table 1): Grade 0 – no bacteria; Grade 1 1–5 bacteria in any field of view; Grade 2 3–10 bacteria in any field of view, isolated or as a cluster; Grade 3 1–2 or more clusters of bacteria in any field of view (25 or more bacteria in each).

BACTERIOLOGICAL STUDY

During autopsy, heart blood and samples from the organs (Table 2) were taken by using the aseptic technique. Heart blood and the organs' homogenate were plated onto blood agar. The blood agar plates were incubated for 48 h at 37°C. Bacterial counts log CFU/ml or /g were calculated.

METHOD OF IMMUNOHISTOCHEMISTRY

Samples of the liver were fixed in 4 % neutral-buffered paraformaldehyde for 24 h. From paraffin-embedded tissue sections slices of 3–4 µm, were cut, put on polysine ™(Menzel Glases) or (Star Frost/Knittel Glases) microscope slides, dewaxed and rehydrated.

Macrophage activity was studied in the liver applying immunohistochemical staining by the Avidin-Biotin method. The slides were washed with Tris-Buffered Saline (TBS) pH 7.4, incubated with proteinase K (Sigma ST Louis, USA), treated with the solution of 5 % bovine serum albumin (BSA) and incubated with Mouse Monoclonal Antibody ED_1 (Nordic BioSite AB) as a primary antibody 1: 50 in TBS and biotinylated Anti Mouse IgG(H + L) (Vector Laboratories) in TBS 1:200 as a secondary antibody, incubated with streptavidin – AP (Vector Laboratories) 1:1000 in TBS. The tissue pieces were colored with BCIP-NBT (Vector Laboratories) in the dark, to observe the reaction. The marked macrophages were counted with the light microscope (ocul. 10×, obj. 40×) in 168 squares, with the side of a square being 23 μm and the whole area 88.872 μm^2.

DETECTION OF APOPTOTIC CELLS

From the same tissue sections of the liver used by us for light microscopy and immunohistochemistry examination, we cut 3–4 μm thick slices, put them on (Star Frost Knittel Glasses) microscope slides, dewaxed and rehydrated. For the detection of apoptotic cells the "In Situ Cell Death Detection Kit" (Roche Molecular Biochemicals) was used applying the reagents and protocols supplied by the kit. The slices were incubated with proteinase K, PCR Grade (Roche Molecular Biochemicals) and washed with PBS pH 7,4. With the kit DNA labeled with deoxyuridine triphosphate (dUTP) fluorescein via action of terminal deoxynucleotidyl transferase (TdT), which attaches dUTP to 3'-OH-DNA was used. Apoptotic nuclei have "nicks" (strand breaks) in their DNA, which are labeled with fluorescein dUTP. Apoptotic nuclei appear to be green. Nuclei without strand breaks are not labeled. Apoptotic cells were counted with the microscope AxioPhot 2 by fluorescent terminal (magnification ocul. 10×, obj. 63×). Total counts of apoptotic cells in 100 microscopic viewing fields of the tissue sections in each microscopic slide were taken. The area of one microscopic field was 35810 μm^2.

STATISTICAL ANALYSIS

Data were analysed by the STATISTICA 6,0 program. Unpaired t-test was used for the comparison of groups with normally distributed data. The Mann-Whitney rank SUM test was used for the comparison of groups without normal distribution. Differences in proportions were compared by using the χ^2 test. Statistical significance was defined as $p \leq 0{,}05$.

RESULTS

All the rats involved in the experiment survived the first six hours after inoculation. The highest mortality – 18 % of the animals – was registered between 6–24 hours, 8 % died during 24–48 hours, and 4 % between 48–120 hours during late sepsis. The applied model of experimental Gram-negative sepsis revealed massive dissemination of E. coli into the investigated organs showing a rapid translocation from the peritoneal cavity.

HISTOLOGICAL EVALUATION OF E. COLI IN TISSUES

All the 12 investigated organs of experimental animals (except the control group) were contaminated with a large number of E. coli foci. No bacteria were found in control animals. The bacteria had formed colonies, located mostly intravascularly, in intestitial spaces, inside macrophages and parenchymal cells (Figs. 1, 2). But polymorphonuclear infiltration around the colonies was not typical in all animals. At the same time, significant tissue damage was seen

TABLE 2

The groups of animals and the samples collected. Bacteriological studies: heart blood (HB), mesenteric lymph nodes (MLN), liver (LI), lungs (LU), spleen (SP), kidney (KI), heart muscle (HM).

Group	Time of killing	Samples
Control	2 h, 6 h, 24 h, 48 h, 5 days	HB, MLN, LI, LU, SP, KI, HM;
I	2 h	HB, MLN, LI,
II	6 h	HB, MLN, LI,
III	24 h	HB, MLN, LI,
IV	48 h	HB, MLN, LI, LU, SP, KI, HM;
V	5 days	HB, MLN, LI, LU, SP, KI, HM;

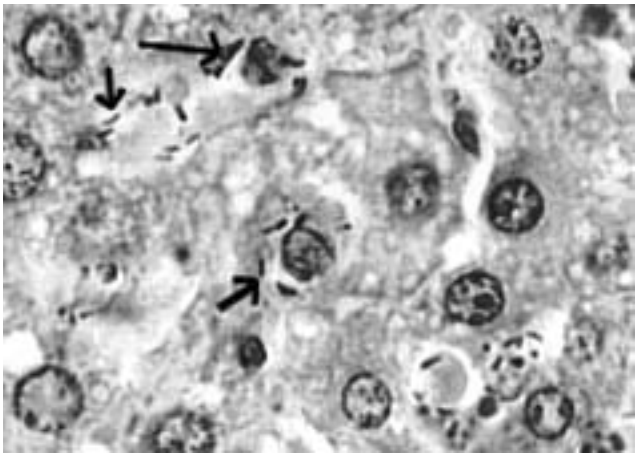

Fig. 1. The clusters of bacteria in the wall of a sinusoid, in the perivascular space and in a hepatocyte (modified Gram's × 900).

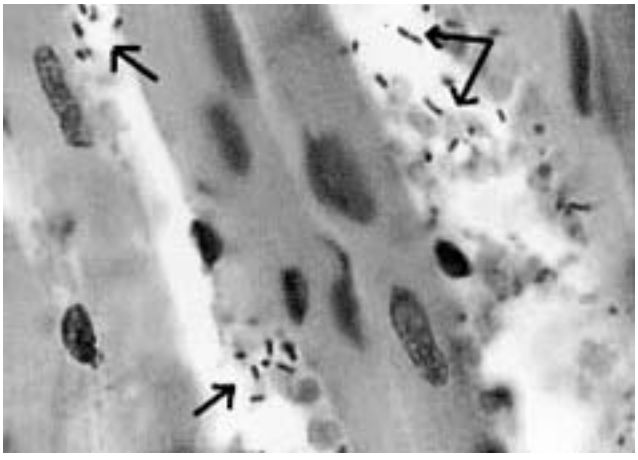

Fig. 2. The clusters of bacteria in the interstitial tissue of the heart (modified Gram's × 900).

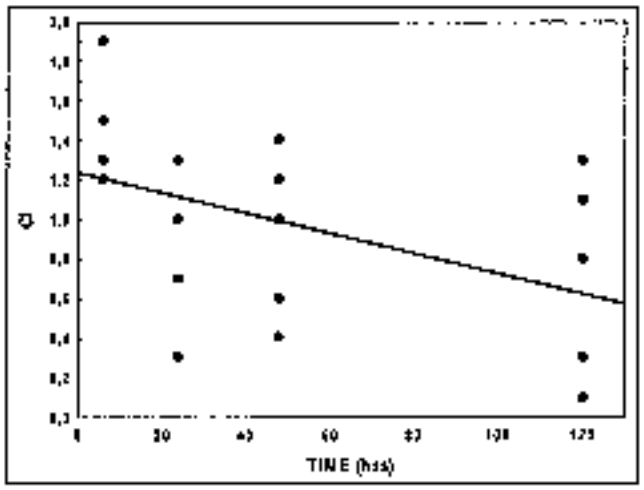

Fig. 3. Correlation between the total mean CI and duration of sepsis ($r = –0.5$, $p < 0.05$).

around the foci. The results of microscopical investigation are presented in Table 1. The mean of the contamination index (CI) was significantly higher in the lungs, spleen, liver and kidney than in the brain, adrenals, pancreas and muscles. The total mean CI of all organs was different between groups. Negative correlation was found between the total mean CI and the duration of sepsis ($r = –0.5$; $p < 0.05$) at the beginning of 6th hour (Fig. 3).

QUANTITATIVE EVALUATION OF THE COUNTS OF E. COLI IN HEART BLOOD AND IN ORGANS

Bacteriological evaluation

The contamination of blood and tissues with E. coli has shown a rapid elevation between 2–6 hours after inoculation (Fig. 4). The first cases of translocation were detected 45 min after inoculation in our earlier work and were found mainly in the liver and mesenteric lymph nodes (10). In the present work we

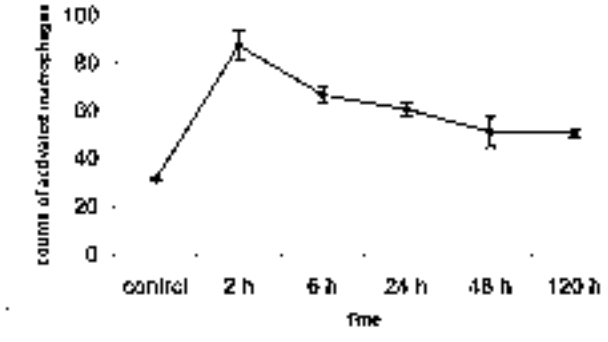

Fig. 5. The counts of activated macrophages in the liver (range I and median •). Statistically significant differences: group I versus control ($p < 0.0002$), group I versus group II ($p < 0.04$).

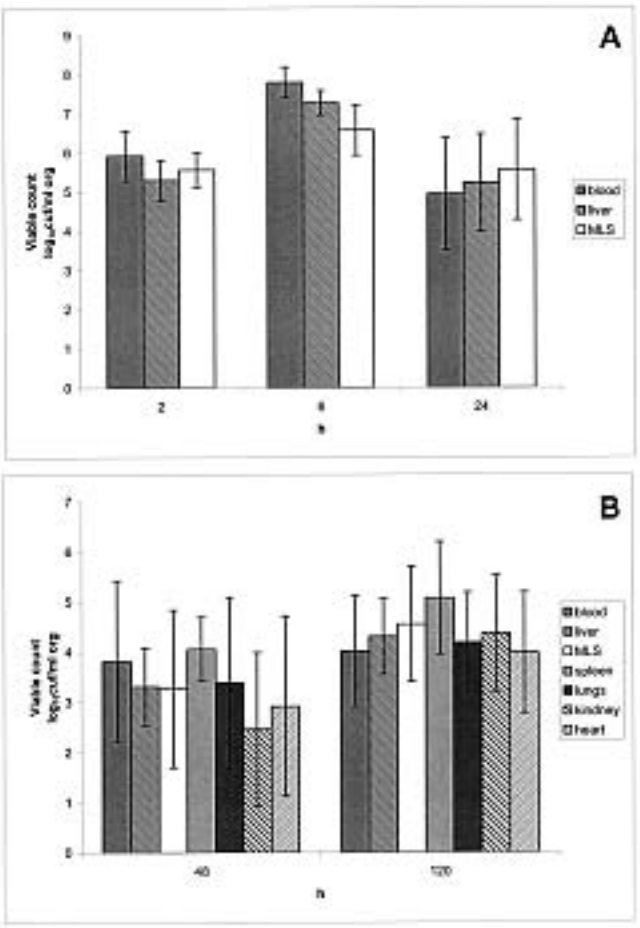

Fig. 4. The counts of E. coli in the heart blood and in the organs (range I and median): A – in early sepsis; B – in late sepsis.

examined the animals 2 h after the onset of sepsis and received the highest counts of E. coli in heart blood and the tissues of the liver and mesenteric lymph nodes 6 h after the onset, that is during early sepsis. After that the counts of E. coli began to fall, reaching the lowest values by 48th h after inoculation into blood and tissues. It was important to establish the fact that a new marked wave of the elevation of the counts of microbes was observed 120 h after inoculation inside the organs – in the liver, kidney, heart muscle, mesenteric lymph nodes, while an increase in the microbes in blood was not observed in all experimental animals, those counts even showing some tendency to decrease in comparison with tissues (Fig. 4).

QUANTITATIVE EVALUATION OF ACTIVATED MACROPHAGES (IMMUNOHISTOCHEMICAL METHOD)

The number of activated macrophages showed the maximum high level at the end of 2nd h after inoculation. After that the number of activated macrophages began to fall, having dropped 1.5 times by 6th hour and continued to fall during the next hours, remaining essentialy higher in comparison with the control group at the end of the experiment (Figs. 5, 6).

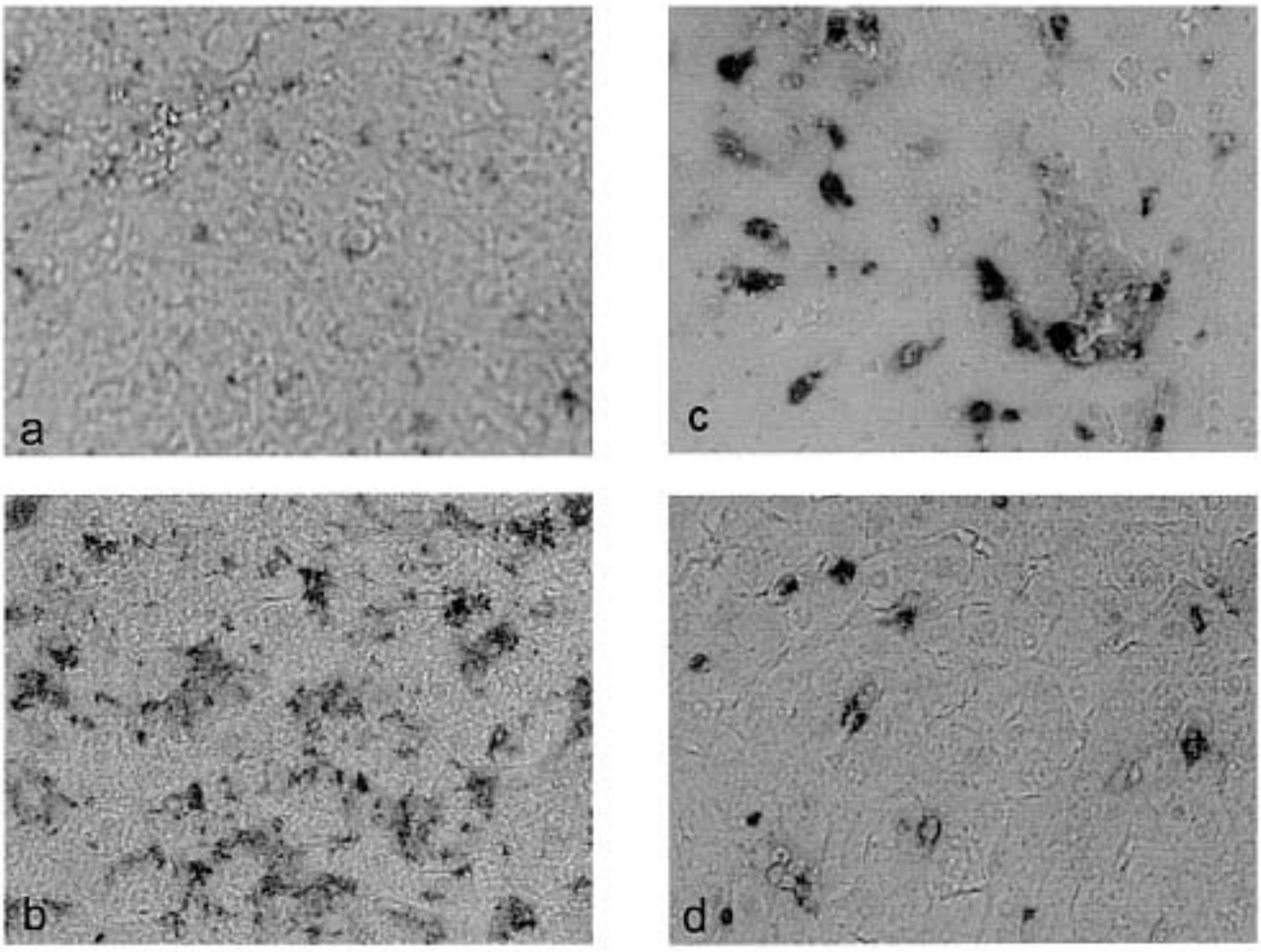

Fig. 6. Macrophages in liver staining by Avidin-Biotin Complex method, using monoclonal antibody ED1. (a) control, (b) 2 h, (c) 6 h, (d) 24 h after inoculation. Magnification (× 400).

QUANTITATIVE EVALUATION OF THE COUNTS OF APOPTOTIC MACROPHAGES (IMMUNOHISTOCHEMICAL METHOD)

Highest counts of apoptotic macrophages were found 6 h after inoculation and showed a rapid fall during the next hours, achieving an equal level with the control group at the end of 5 days. During the first two hours the number of apoptotic macrophages did not show a marked increase. A sharp increase followed after two hours, achieving the highest values by 6th hour. At the end of 6th h 18.5 % of all macrophages were apoptotic, at the end of 24th hour 10.6 %, and at the end of 48th h 8.8 % of all macrophages were apoptotic. The apoptotic cells appeared to be mainly the Kupffer cells (Figs. 7, 8), as well as some lymphocytes and neutrophils.

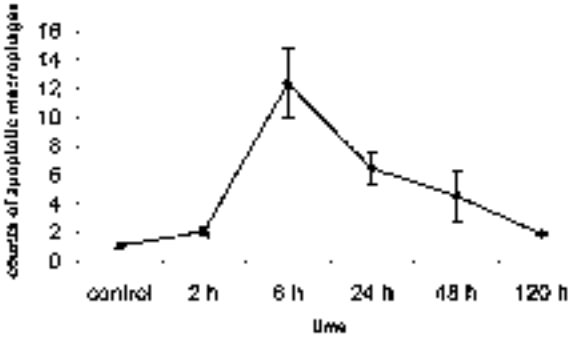

Fig 7. The counts of apoptotic macrophages in the liver (range I and median •). Statistically significant differences: group I versus group II ($p < 0.004$), group II versus group III ($p < 0.02$).

CORRELATION BETWEEN THE COUNTS OF E. COLI IN HEART BLOOD AND IN DIFFERENT ORGANS

In early sepsis the counts of E. coli in blood and in the organs were positively correlated. The highest values of both parameters were found 6 h after inoculation when strong positive correlations were found ($p < 0.001$). Correlation coefficients (r) were: the liver $r = 0.95$; mesenteric lymph nodes $r = 0.91$; the lungs $r = 0.94$; the lien $r = 0.96$; the kidney $r = 0.96$; the heart $r = 0.94$.

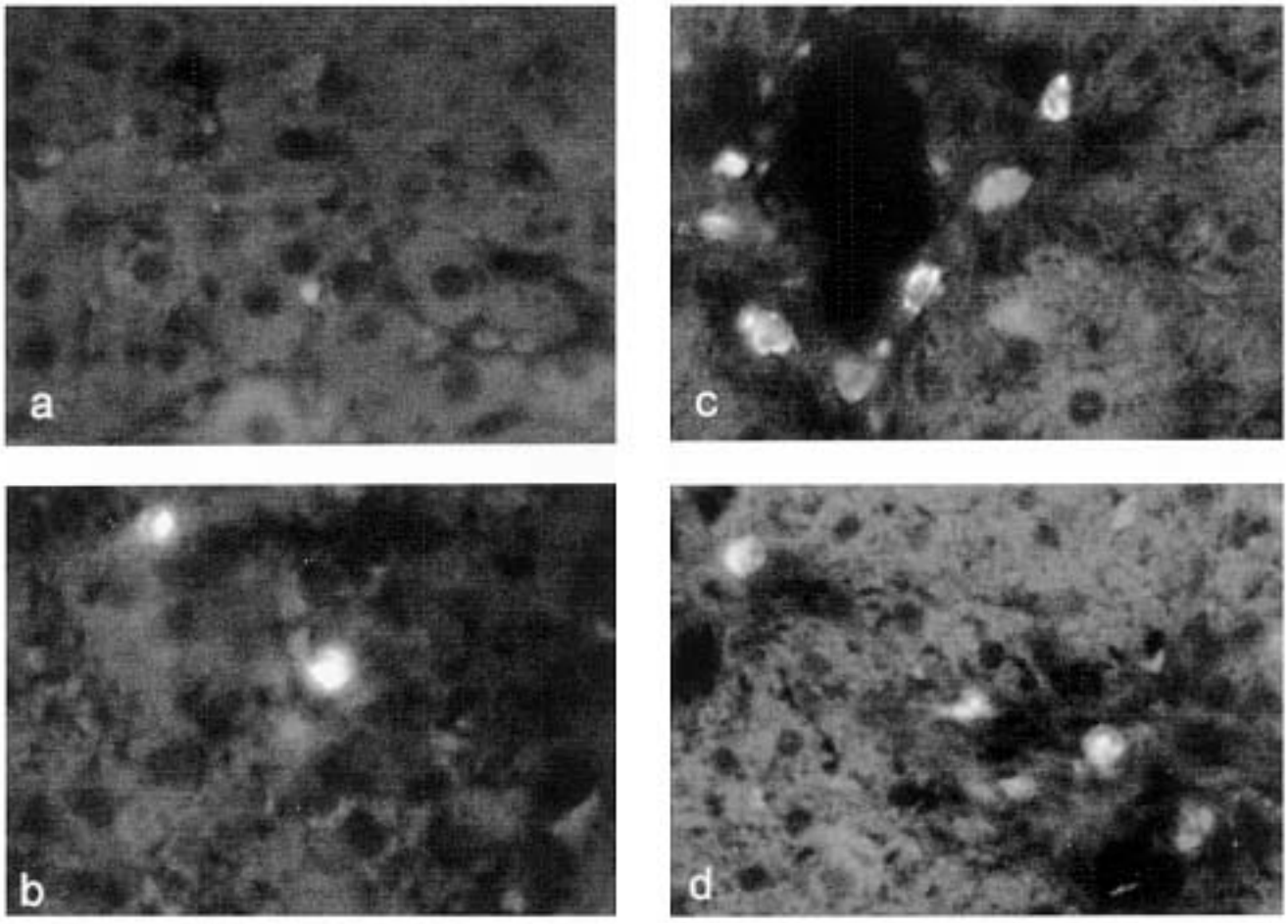

Fig. 8. Fluorescence photomicrographs of apoptotic macrophages in the liver. (a) control, (b) 2 h, (c) 6 h, (d) 24 h after contamination. Magnification (× 700).

CORRELATION BETWEEN THE COUNTS OF E. COLI IN HEART BLOOD AND THE COUNTS OF ACTIVATED MACROPHAGES IN THE LIVER

In early sepsis the number of the counts of microbes in blood was negatively correlated with the number of activated macrophages. At the end of 6th h the counts of microbes in blood revealed the highest value, while the number of activated macrophages had essentially fallen, showing a negative correlation (r = –0.6).

CORRELATION BETWEEN THE COUNTS OF E. COLI IN HEART BLOOD AND THE COUNTS OF APOPTOTIC MACROPHAGES IN THE LIVER

In early sepsis, at the end of 6th h, the highest counts of microbes in heart blood were positively correlated with the highest counts of apoptotic macrophages (r = 0.98).

DISCUSSION

The model of experimental sepsis that was used revealed massive dissemination of E. coli from the peritoneal cavity into blood and multiple organs. Evaluation of the counts of E. coli in the organs by using both the histological method (12 organs studied) and the bacteriological method (5 organs) revealed massive translocation during early sepsis. Certain difference was seen during the first hours as well as during late sepsis. Bacteriological evaluation seems to be a more exact method revealing an earlier translocation into tissues already 45 min after inoculation and a late new wave of contamination after 5 days.

In our earlier clinical and experimental studies a correlation was detected between the duration of sepsis and the severity of histopathological changes. The morphological changes of various severity and extent were present in all investigated organs by 24th h, however, high variability between the animals occurred. The vascular changes of different frequency such as hyperaemia, intestinal and perivascular oedema and stasis found in most of the organs by 24th hour were accentuated in the lungs, liver and kidneys. More severe changes of a different expression – haemorrhages, thrombi, necrosis – prevailed in late sepsis (12, 14). Vascular changes seem to be possible reasons which led to the obstruction of blood flow already during the first hours and, owing to that, to the destruction and necrosis of tissues. The counts of E. coli having a strong positive correlation between

blood and tissues during early sepsis were not in such a strong association during late sepsis, being higher in tissues than in blood in some groups of animals. Surprisingly, the second wave of the multiplication of microbes was revealed in tissues 120 h after inoculation in bacteriological estimations (in some animals also in histological examination) (Fig. 4). The following dynamics of contamination in late sepsis would be of great interest for future investigations. That assured us of the existing "two hit" phenomenon of sepsis (15, 16), clinically thought to be due to the priming and activation sequence where the first stress enhances susceptibility to the second stress by priming inflammatory cells (16). The second rise of microbes by 120th h in tissues did not reflect the same tendency in our results in the blood of all animals. This suggests different rates of bacterial multiplication or clearance in blood and tissues, which may also be dependent on the individual reaction of the host. We suggest and support the theory that this difference is most probably caused by the disturbances of microcirculation. Due to various reasons capillary stasis and the plugging of microvessels occurs. In the sites of obstruction or a very slow blood flow, deficiency of phagocytosis and uncontrolled growth of microbes may be present (17, 18, 19, 20, 21). Our findings of bacterial clusters in capillaries support this suggestion (11, 12). The second wave of the multiplication of microbes proved our supposition that it must take hours of stasis in microcirculation to enable bacteria to multiply in this amount in tissues. The mechanisms of bacterial clearance in blood and tissues are probably different. In the serum humoral factors, e.g. the complement system, are more important, while in tissues cellular mechanisms, mostly macrophage activity, is responsible for the killing of bacteria (22).

In accordance with our hypothesis, the septic shock and MOF (Multiple Organ Failure) initiated by an excessive response of the host to infection, involving the activation of inflammatory cells, such as macrophages, monocytes and neutrophils (2, 7, 23), we focused our interest on the activation of macrophages in the liver in different stages of sepsis. One explanation would be that Gram-negative infection reduces macrophage activity in the acute phase to such an extent that might be generally accompanied by a decrease in immunoreactivity (24). The low bacterial clearance rate in tissues might be due to low macrophageal activity as the macrophageal reaction was not typical around microfoci. This finding is contradictory to the idea of generalised and massive activation of macrophages in sepsis (23). Our data reveal the fact that the activation of macrophages is an early response to infection during the first hours after inoculation, showing the highest level at the end of 2nd h after inoculation. By 6th h the number of macrophages had fallen 1.3 times and showed a continuous decrease. The highest counts of microbes in the organs and heart blood were found to be strongly related to the low counts of activated macrophages. These data coincide with the result of Ayala and other investigators who reported that macrophages were activated during the early 0–4 h after the experimental septic insult to act as sources of proinflammatory cytokines (6, 7). Sepsis is reported to be associated with the decreased rate of macrophage function and the increased inducible macrophage apoptosis, which recognize and ingest cells undergoing apoptosis (6, 25). A number of laboratories have stated that sepsis induces a marked suppression in both innate and acquired immune responsiveness (26, 27). Although the majority of investigators indicate that sepsis appears to be associated with an increased rate of apoptosis (26, 27), according to other researchers it is not possible to claim definitely that sepsis is the cause of extensive apoptosis, and that the extent of cell death is not generally sufficient to cause organ failure (5, 24). Our findings described above revealed a marked increase in activated macrophages by 2nd hour after inoculation, which is associated with the lowest values of apoptotic cells. The depressed macrophage response during the following hours appears to be associated with the increased rate of apoptotic cells, which reaches the maximum level 6 hours after inoculation. These data support the result of Ayala and others (5, 25, 27). The highest count of apoptotic cells was found 6 h after inoculation, having a strong positive correlation with the highest counts of microbes in tissues and blood. The marked elevation of apoptotic cells seems to be in strong correlation with the depression of macrophage activity. A number of studies and our data suggest that the high level of activated macrophages 2 h after inoculation may be caused by bacterial endotoxins, which is probably the reason for early morphological abnormalities in tissues. The consequent high level of apoptotic cells was associated with inflammatory cytokines produced by activated macrophages (6, 16, 26).

A number of studies have suggested that macrophage cytokines primarily induced by endotoxin, a hypoproduct of the breakdown of Gram-negatative bacteria, serve as the agents that initiate developing cell and organ dysfunctions during sepsis (16, 17). In conclusion, the massive penetration of bacteria into tissues was present in multiple organs. The depressed macrophage response in early sepsis following the increased rate of apoptotic macrophages, and the second wave of the multiplication of bacteria in tissues in late sepsis all refer to immunosupression, which produces a general tissue damage in the body. Different rates of bacterial multiplication or clearance in blood and tissues in early and late sepsis, the possible second wave of multiplication of bacteria need to be carried out in further works.

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