MODELS OF INFECTION

Translocation of indigenous microflora in an experimental model of sepsis

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Translocation of viable bacteria from gut to bloodstream and other sterile body sites during shock has been demonstrated in several experimental and clinical studies. The factors causing translocation and its incidence at different stages of shock are not known. The aim of the study was to evaluate the importance of several factors causing translocation of indigenous microflora in an experimental model of septic shock based on intraperitoneal *Escherichia coli* sepsis in rats. Counts of inoculated *E. coli* and translocated bacteria in different locations, gut morphology and haematological values were evaluated at different stages of sepsis. Sepsis developed in all animals and *E. coli* achieved the highest counts in blood 6 h after inoculation. Translocation was commonest at 6 and 12 h after inoculation. Frequently translocating bacteria were lactobacilli, bifidobacteria, bacteroides and peptostreptococci. In early sepsis, translocation was associated with high *E. coli* counts in blood, yet in late sepsis the opposite correlation was present. Low infiltration by neutrophils in the ileum and decreased mitotic activity in the colon were associated with a high translocation rate. In early sepsis, translocation was associated with low lymphocyte counts, but in late sepsis, with low neutrophil counts. Translocation of bacteria (including anaerobes) that colonise the gut in high counts takes place during sepsis. Putative influencing factors such as activity of the primary disease (bacterial counts in blood), gut morphology or haematological values seem to have different impacts on translocation, depending on the stage of the disease.

Introduction

Bacterial translocation is the passage of viable indigenous bacteria to sterile body sites, such as the mesenteric lymph nodes (MLN), spleen, liver and bloodstream [1]. Although bacterial translocation can take place through several mucosal membranes, most interest has focused on the intestinal tract as potentially the most important source of translocating bacteria [1–4]. It is generally agreed that gram-negative facultative bacteria translocate more easily than anaerobes and gram-positive bacteria [5].

Several factors – such as bacterial overgrowth, alteration of mucosal barriers and immunosuppression – promote bacterial translocation from the intestinal tract [6]. An imbalance of intestinal microflora and overgrowth by gram-negative facultative bacteria through administration of antibiotics enhances translocation

[1, 7]. Damage of the mucosal barrier by several factors, including haemorrhagic- and endotoxin-induced shock, can also induce bacterial translocation [8, 9]. However, evidence for the correlation of translocation with intestinal mucosal permeability to non-bacterial compounds is still contradictory [10, 11].

Despite many experimental and clinical studies, it is not known whether indigenous bacteria translocate with equal efficiency from all sites of the gastrointestinal tract or whether they translocate more efficiently from certain regions [1]. Most studies investigating a possible relationship between histological changes in mucosae and translocation have focused on changes in the small bowel and only a few studies have analysed and demonstrated alterations in colonic mucosa [12, 13]. Passage of bacteria through mucosal membranes and survival in extra-intestinal sites depend on the effectiveness of the host immune system. Both humoral and cellular immunity have been shown to be important in protection against translocation [1, 2, 14]. However, it is not known if one of them prevails at different stages of bacterial translocation.

Thus, several physiological and pathological conditions could be associated with enhanced translocation of indigenous bacteria through mucous membranes. Although the presence of bacterial translocation during shock and other serious diseases has been demonstrated clearly in several experimental and clinical studies, the real incidence of translocation in different stages of disease, the factors to which it is related, and the influence of translocation on patient outcome are still unclear [4, 8, 15, 16].

The aim of the study was to detect the translocation of indigenous bacteria during early and late *Escherichia coli* sepsis in an experimental animal model and to correlate it with several sepsis-associated parameters such as *E. coli* counts in blood, histological changes in intestinal mucosa and haematological values.

Materials and methods

Experimental animals

Wistar rats (n = 51) were challenged with an *E. coli* isolate from the blood of a septic patient. Viable *E. coli* cells (2×10^7/g of body weight) were suspended in 1.5 ml of saline and inoculated intraperitoneally together with 1.5 ml of haemolysed blood. Blood was added to suppress an initial immune response in the peritoneal cavity [17]. Fourteen rats died before the end of the study and were excluded from the analysis. A total of 37 rats (median weight 241 g) was included in the study and some were killed 45 min (group I), 2 h (group II), 6 h (group III), 24 h (group IV), 48 h (group V) or 5 days (group VI) after inoculation (Table 1). A further seven uninoculated rats served as controls. The rats had free access to standard laboratory chow and water *ad libitum* during the study, which was approved by the local ethical committee of animal research.

Processing of bacteriological samples

During autopsy, heart blood and samples from the mesenteric lymph nodes, liver, spleen, lung, heart muscle and kidney were taken. The organs were weighed, homogenised in phosphate buffer, serially diluted in an anaerobic chamber (Sheldon Manufacturing) and seeded on: Wilkins Chalgren Blood Agar (Oxoid, CM619) for isolation of anaerobes; Blood Agar (Oxoid, CM271) for isolation of aerobes; Wilkins Chalgren Agar with vancomycin and nalidixic acid (Oxoid, CM619, SR108) for selective isolation of gram-negative anaerobes and Wilkins Chalgren Agar with colistin and nalidixic acid (Oxoid, CM619, SR70) for selective isolation of gram-positive organisms. Blood (125 μl) was inoculated into broth (Signal Blood Culture System, Oxoid) and another 125 μl were seeded on Wilkins Chalgrin blood agar. For quantitation of bacteria, serial dilutions of blood and peritoneal fluid were seeded from the above-mentioned selective and non-selective media. Blood agar plates were incubated aerobically for 48 h at 37°C. Plates of the other media were incubated at 37°C in an anaerobic chamber for 5 days. After incubation, the colonies were counted and colonies of different morphotypes were isolated for identification to the genus level. Bacterial counts/g or /ml and the number of translocation cases (i.e., the sum of different organ/microbe combinations) were calculated.

Processing of histological samples

Samples from the central part of the ileum and the colon (3 cm from anus) were taken and fixed with neutral formalin 10%. Paraffin sections were stained with haematoxylin and eosin, acridine orange and a modified Gram's stain. Two experienced histopathologists examined coded slides blind. Several inflammatory and destructive changes such as: hyperaemia, haemorrhages, vacuolisation, necroses, goblet cell counts, mitotic activity, diffuse lymphatic infiltration, infiltration by polymorphonuclear leucocytes (PMNLs) and the presence of microbes within crypts were evaluated. A numerical score was given to the changes: $0 = $ norm; $-1, -2, -3 = $ diminishing (mild, moderate, severe changes) as compared with the norm; 1, 2, 3 = increasing changes as compared with the norm. The norm was defined by the examination of control rats as in a previous study [18].

Counting of blood cells

Blood samples from all killed animals were collected into standard EDTA-coated test tubes. Total counts of white blood cells (WBC) and lymphocytes were measured in a Sysmex K 1000 machine (TOA Medical Electronics, Kobe, Japan). Blood smears were made

Table 1. The groups of animals and the samples collected

Group	Time of killing	Samples
Control	2 days	PC, HB, MLN, Li, Sp, Lu, HM, Ki, Co, Il
I	45 min	PC, HB, MLN, Li, Sp, Co, Il
II	2 h	PC, HB, MLN, Li, Co, Il
III	6 h	PC, HB, MLN, Li, Co, Il
IV	24 h	PC, HB, MLN, Li, Co, Il
V	48 h	PC, HB, MLN, Li, Sp, Lu, HM, Ki, Co, Il
VI	5 days	PC, HB, MLN, Li, Sp, Lu, HM, Ki, Co, Il

Bacteriological studies: PC, peritoneal cavity; HB, heart blood; MLN, mesenteric lymph nodes; Li, liver; Sp, spleen; Lu, lung; HM, heart muscle; Ki, kidney. Histological studies: Co, colon; Il, ileum.

and stained according to Pappenheim for counting neutrophils, monocytes and eosinophils.

Statistical analysis

Data were analysed by a Jandel SigmaStat 2.0 program. The following tests were used: unpaired t test for comparison of groups with normally distributed data, Mann-Whitney rank sum test for comparison of groups without normal distribution, Spearman rank order correlation to quantify the relationship between several variables and the χ^2 or Fisher test for prevalence comparison of changes between groups.

## Results

Development of sepsis

The model of infection was suitable for investigation of different stages of sepsis. All inoculated animals survived at least 6 h; during the following days a lethal infection developed in some animals, but others survived. The survival rate of the animals over 5 days was: 100% at 6 h; 85% at 1 day; 77% at 2 days and 62% at 5 days. All control animals remained asymptomatic. Because different trends were found in several parameters of animals killed at different times after inoculation, the animals were grouped into those with early sepsis (45 min–24 h) and those with late sepsis (2–5 days).

The dissemination of *E. coli* from the peritoneal cavity to sterile sites was detected as early as 45 min after inoculation. The counts of *E. coli* were highest in the blood of animals in group III (6 h after inoculation) (Fig. 1). Significantly lower counts in blood were found in rats from groups I, II, V and VI. Similar trends were seen in the *E. coli* counts in the mesenteric lymph nodes, liver and peritoneal cavity. When comparisons were made between the counts of *E. coli* in blood and

other organs of animals in the same group, it was found that the counts in the peritoneal cavity remained higher than those in blood during early sepsis (median 8.1 *versus* 5.6; $p < 0.001$) and late sepsis (median 7.8 *versus* 5.6; $p < 0.001$). In other organs, the counts of *E. coli* did not differ significantly from those in blood. When the counts in individual animals were correlated, a strong positive association was found between the counts of *E. coli* in blood and in other organs ($p < 0.001$). Correlation coefficients (r) were: mesenteric lymph nodes, $r = 0.79$; liver, $r = 0.85$; peritoneal cavity, $r = 0.83$, spleen, $r = 0.83$; lungs, $r = 0.88$; kidney, $r = 0.9$; heart muscle, $r = 0.84$.

Translocation of normal microflora to sterile body sites

No cases of translocation were present in the control group. In the test group only a few cases of translocation were detected 45 min after inoculation (group I animals) (Table 2). In group VI animals (5 days after inoculation) translocation was also relatively rare. Bacterial translocation was most frequent in groups II–V, i.e., in animals 2–48 h after inoculation with *E. coli*. In early sepsis, the highest incidence of translocation was seen in the mesenteric lymph nodes. In late sepsis, bacteria were found most frequently in kidney and lungs. Indigenous bacteria were recovered from blood in only a few cases. The most commonly translocating bacteria were lactobacilli, bifidobacteria, bacteroides and peptostreptococci which were found together in 63% of all cases of translocation (Fig. 2). The least frequently translocated bacteria were enterococci, staphylococci, streptococci, propionibacteria, eubacteria and gram-negative non-fermenters. If translocation was present, one or two bacterial groups were usually recovered from the same organ in early sepsis. In late sepsis, translocation varied more between individual animals: in some rats no translocation to any organs was detected, but in others up to seven
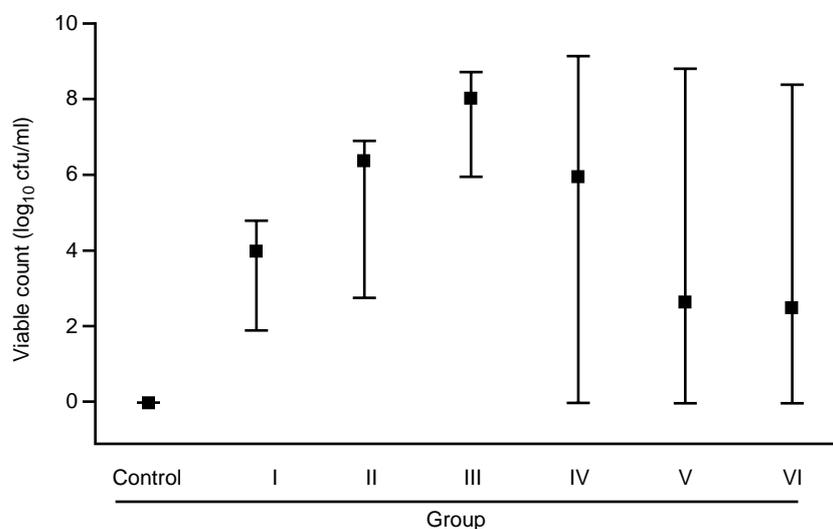
Fig. 1. The counts of *E. coli* in heart blood (range | and median ■). Statistically significant differences: group I *versus* II ($p < 0.05$); group I *versus* III ($p < 0.001$); group III *versus* V ($p < 0.05$); group III *versus* VI ($p < 0.05$).

Table 2. The frequency of bacterial translocation and the organs involved

| Organ | Number of animals with/without translocation | | | | | |
	Group I	Group II	Group III	Group IV	Group V	Group VI
Blood	0/6	1/5	1/5	0/6	1/4	0/8
MLN	2/4	6/0	6/0	5/1	4/1	2/6
Liver	1/5	4/2	5/1	4/2	3/2	0/8
Spleen	0/6	ND	ND	ND	1/4	0/8
Lung	ND	ND	ND	ND	4/1	4/4
Heart muscle	ND	ND	ND	ND	3/2	1/7
Kidney	ND	ND	ND	ND	2/3	4/4

ND, not determined. No cases of translocation were found in the control group. Significant differences between groups: MLN, II *versus* VI ($p = 0.01$), III *versus* VI ($p = 0.01$); liver, I *versus* III ($p < 0.05$), VI *versus* II ($p < 0.05$), VI *versus* III ($p < 0.01$), VI *versus* IV ($p < 0.05$), VI *versus* V ($p < 0.05$).

different bacterial groups were isolated from the same organ. The counts of indigenous bacteria from normally sterile organs were up to $\log_{10}$ 4.6 cfu/g.

Histological studies of intestinal mucosa

There were no histological changes in the mucosal samples of the control animals except for a mild reduction in mitotic activity in one rat. At 45 min after inoculation (group I animals), only mild changes (reduction of mitotic activity and infiltration by lymphocytes and PMNLs) were seen in a few rats. In the group II rats (2 h after inoculation) deviations from the norm were more pronounced. In most of these animals hyperaemia, vacuolisation, reduction of goblet cells, decreased mitotic activity and infiltration by lymphocytes and PMNLs were detected. However, the expression of these characteristics in individual animals within the groups was highly variable. Because of this, no statistical difference between groups II–VI (as well as early and late sepsis) could be demonstrated. In all group II–VI rats, different morphotypes of bacteria were found within the base of the crypts of the ileum (Fig. 3). In control and most group II animals, bacteria were not detected in the crypts.

In comparisons of particular changes in the ileum and colon of individual animals a higher reduction of goblet cells ($p < 0.05$) was found and a more pronounced lymphatic infiltration ($p < 0.005$) in the ileum. No significant differences were detected between the ileal and colonic mucosa in the prevalence or intensity of other characteristics.

Correlation between the counts of E. coli, translocation of indigenous microflora and histological changes in mucosae

Correlation between the counts of E. coli and histological changes in mucosae. In early sepsis, *E. coli* counts in blood correlated positively with several particular and total mucosal changes in the ileum (Table 3). Correlation with changes in the colon was weaker. In late sepsis, the association between *E. coli* counts in blood and changes in the ileum was weak and not statistically significant. In the case of the colon, the association with most histological characters was weakly negative, but the total score of changes in the colon gave a significant negative correlation.

Correlation between the counts of E. coli and translocation of indigenous bacteria. In early sepsis, the counts of *E. coli* in blood were positively associated with total counts of translocated bacteria to primarily sterile organs (Fig. 4a) as well as the number of translocation cases ($r = 0.66$, $p < 0.001$). In late sepsis the opposite trends were found. There was a negative correlation between the counts of *E. coli* in blood and the total counts of translocated bacteria (Fig. 4b).

Correlation between translocation of indigenous bacteria and histological changes in mucosae. In early sepsis, the number of translocation cases was negatively associated with mitotic activity in the colon ($r = -0.71$, $p = 0.001$) and positively with the presence of microbes in the crypts of the colon ($r = 0.52$, $p < 0.05$) and a total score of histological changes in the colon ($r = 0.52$, $p < 0.05$). The total counts of translocated bacteria were in negative correlation with mitotic activity in the colon ($r = -0.5$, $p < 0.05$).

In late sepsis a significant negative association was found only between infiltration of PMNLs in the ileum and the number of translocation cases and counts of bacteria translocated into blood, MLN and liver (in both cases $r = -0.67$, $p < 0.05$).

Correlating translocation and the histology of mucosae of all infected animals, it was found that higher incidences of translocation cases were significantly associated with decreased mitotic activity in the colon ($r = -0.5$, $p < 0.01$) and lower infiltration by PMNLs in the ileum ($r = -0.62$, $p < 0.05$).

Correlation of E. coli counts and translocation with haematological values. A significant negative correlation was found between the counts of *E. coli* in blood and the total count of white blood cells ($r = -0.96$, $p < 0.001$). This association was present in early ($r = -0.64$, $p < 0.005$) as well as late sepsis ($r = -0.79$, $p < 0.05$). A similar association was
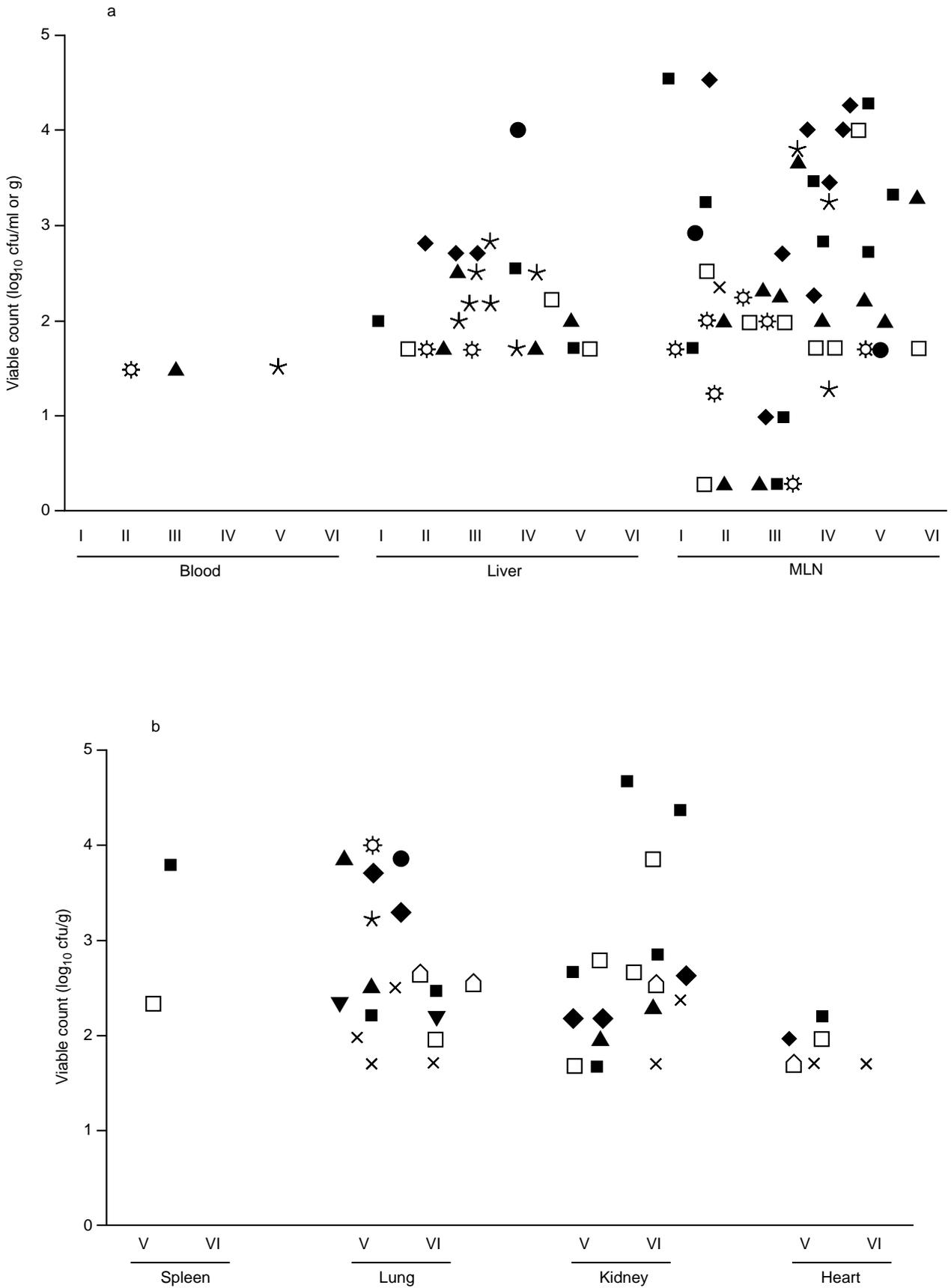
Fig. 2. (**a**) Bacterial translocation of indigenous microflora to blood, liver and mesenteric lymph nodes (MLN) in rats of groups I–VI. Lactobacilli ■; bifidobacteria □; bacteroides ▲; peptostreptococci ◆; coliforms ✳; enterococci ✿; staphylococci ✕; streptococci ●. (**b**) Bacterial translocation of indigenous microflora to spleen, lung, kidney and heart in rats of groups V and VI. Lactobacilli ■; bifidobacteria □; bacteroides ▲; peptostreptococci ◆; coliforms ✳; enterococci ✿; staphylococci ✕; streptococci ●; glucose non-fermenting gram-negative organisms ▼; propionibacteria △.

Fig. 3. Different morphotypes of bacteria (arrow) found in the connective tissue at the base of the crypts of ileum (modified Gram's stain ×900).

Table 3. Significant correlations between *E. coli* counts and histological changes in mucosae

Histological changes	Correlation coefficient/p		
	Early sepsis	Late sepsis	All
Hyperaemia in ileum	0.47/<0.05	NS	NS
Haemorrhage in ileum	0.53/<0.05	NS	NS
Necrosis in ileum	0.54/<0.01	NS	NS
Microbes in crypts of ileum	0.47/<0.05	NS	NS
Microbes in crypts of colon	0.62/<0.01	NS	NS
Infiltration with PMNLs in ileum	NS	NS	0.43/<0.05
Total score of ileum	0.68/<0.01	NS	0.44/<0.05
Total score of colon	NS	−0.6/<0.05	NS
Total score of ileum and colon	0.60/<0.05	NS	NS

NS, not significant.

present between the counts of *E. coli* and lymphocytes in blood ($r = -0.79$, $p < 0.001$).

Correlating haematological values with translocation, it was found that in early sepsis the number of translocation cases was negatively associated with the counts of lymphocytes in blood ($r = -0.7$, $p < 0.05$), but in late sepsis with the counts of neutrophils ($r = -0.65$, $p < 0.01$).

Discussion

Many different experimental models have been developed to determine the rate and importance of bacterial translocation. In most models, translocation has been detected at one or two fixed times after a single challenge or alteration, e.g., injection of endotoxin. In the rat model used in the present study, the continuous liberation of endotoxin and modulation of the immune response take place during development of sepsis. Translocation to several organs and histological changes in different intestinal sites were determined at different times during early and late sepsis. Although the interpretation of these results is probably more complicated, we believe that this model provides a better reproduction of the actual clinical development of sepsis.

E. coli translocated rapidly from the peritoneal cavity to blood and other organs within 45 min and achieved the highest counts after 6 h. In the primary inoculation
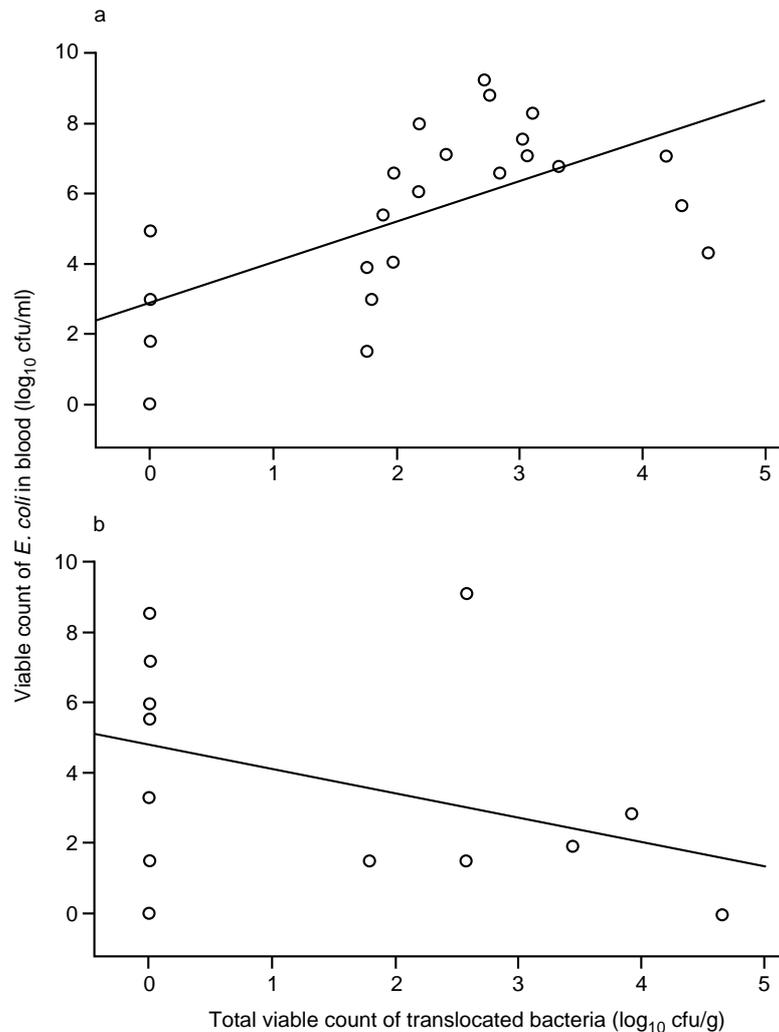
Fig. 4. (a) Correlation ($r = 0.62$; $p = 0.001$) between the counts of *E. coli* in blood and counts of translocated bacteria in early sepsis. (b) Correlation ($r = -0.59$; $p = 0.03$) between the counts of *E. coli* in blood and counts of translocated bacteria in late sepsis.

site – the peritoneal cavity – the counts of *E. coli* were significantly higher than those in blood and other organs throughout the whole experiment. There was a strong positive correlation between the counts of *E. coli* in the peritoneal cavity and in blood. This indicates that the most active proliferation of bacteria takes place in the primary infection site. From this site bacteria disseminate to blood and almost equally to other organs.

High counts of *E. coli* were found to be strongly associated with low counts of lymphocytes and total WBC. While on the one hand, the high counts of *E. coli* and subsequent liberation of high amounts of endotoxin and inflammatory mediators lead to a more pronounced dysfunction and destruction of immune cells [19], on the other hand, higher counts of WBC and lymphocytes could serve as indicators of a more active immune response, effectively eradicating bacteria from blood.

Gram-negative sepsis led to massive bacterial translocation, mainly to MLN and the liver, that started as early as 45 min after inoculation and achieved the highest incidence between 2 and 24 h after challenge with bacteria. In early sepsis, the translocation to MLN and liver was present in most animals, whereas in late sepsis individual differences appeared. In some animals, translocation of many different bacteria to several organs was present, but in others, the translocation was absent or only few a species of bacteria were found in one or two organs. Translocating microbes were found to be mainly indigenous anaerobes or facultative bacteria such as lactobacilli, bifidobacteria, bacteroides and peptostreptococci, bacteria that normally colonise the intestine in high numbers. A mainly protective function has been attributed to these bacteria. Although it was not possible to distinguish indigenous *E. coli* from the inoculated strain, the translocation of other coliforms and aerobes was relatively rare. Most previous investigators have attributed the main role in bacterial translocation to aerobic and facultative gram-negative bacteria. Yet, as in this study, some other studies have also shown the high translocation rate of anaerobes [2, 20]. It is possible that there are no major differences between the translocation rates of faculta-

tive and anaerobic bacteria, but that the coliforms survive better in the bloodstream and lymph nodes than anaerobes, as some authors have speculated previously [2, 5]. The massive liberation of endotoxin and inflammatory modulators during development of septic shock probably suppresses the immune response against translocating bacteria and, therefore, even more susceptible bacteria such as anaerobes can survive and cause further septic complications, as seen in this study. If this is true, in clinical cases of severe sepsis and shock, the concepts of selective decontamination of the gut and selection of antibiotic prophylactics and empirical therapy of septic complication during shock should be revised.

Correlating the counts of *E. coli* in blood and translocation (count of translocated bacteria and prevalence) showed that, in early sepsis, high *E. coli* counts were clearly associated with the increase of translocation of indigenous bacteria. Surprisingly, the opposite trend was present in late sepsis. One hypothesis to explain this paradox could be the probable modification of the immune response during development of sepsis. It is known that endotoxin induces liberation of different pro- and anti-inflammatory mediators. In early sepsis, increased permeability of intestinal mucosae to bacterial translocation because of direct action of endotoxin or *via* different mediators leads to massive translocation of bacteria prevailing on mucosae. The finding that counts of *E. coli* in blood correlated well with several changes in mucosae in early sepsis supports this opinion. It is possible that suppression of the immune system enables translocated bacteria to survive. It may be that in late sepsis the presence of *E. coli* in blood induced an immune response against translocating bacteria, or alternatively, translocated bacteria caused beneficial non-specific immunostimulation against *E. coli*. Moreover, at this stage no correlation between the counts of *E. coli* and particular changes in mucosae was seen and the sum of all scores of colon alterations was even negatively associated with *E. coli* counts.

Higher counts of neutrophils in blood were associated with a rarer detection of indigenous bacteria in sterile organs in late but not in early sepsis. It is known that the trapping of immune cells to microcirculation takes place in the early stages of sepsis [21]. Higher counts of neutrophils in blood in late sepsis probably indicated more active reproduction of these cells. In these animals, neutrophils have an important role in killing invading bacteria. Correlation between translocation and dysfunction of cell-mediated immunity has also been shown in other studies [22].

Some morphological abnormalities in intestinal mucosae were present in all infected animals. After 45 min of bacterial challenge, only mild changes in a few characteristics were seen. By 2 h after inoculation, moderate to severe mucosal changes were present.

However, high variability in particular characteristics between animals even within the groups was present. Reduction of mitotic activity, lymphatic infiltration and presence of bacteria within the crypts were the most common changes detected in nearly all infected animals. Previous animal experiments have shown more pronounced changes in the ileum and caecum than in the colon after administration of endotoxin for induction of shock [1]. Surprisingly, a greater reduction of goblet cells and more expressed lymphatic infiltration in the ileum were the only differences found between the changes in ileum and colon.

Correlating changes in mucosae with translocation, no clear association was found between translocation and severe destructive morphological changes in mucosae, such as necrosis and haemorrhage. The decreased mitotic activity that was found in most infected animals correlated with higher levels of translocation. This change was probably one indicator of mucosal dysfunction during endotoxin-derived shock. In early sepsis there was also correlation between translocation and the total score of mucosal changes in the colon, but not in the ileum. These findings are contrary to the opinions of some others that associate translocation with changes in ileal mucosa [1, 12]. On the other hand, the results of the present study indicate that higher counts of PMNLs in ileal mucosa probably have some protective function against translocation. It seems that evaluation of mucosal permeability to bacterial translocation is complex and several immunological factors, including PMNL activity, should be considered beside morphological destruction of cells.

This study revealed that translocation of different indigenous bacteria, including anaerobes, takes place during sepsis. These microbes can cause further polymicrobial septic complications in different organs. Prediction of the intensity of translocation seems to be difficult. Putative factors such as activity of primary disease (bacterial counts in blood) or haematological values seem to have a controversial impact on translocation, depending on the stage of disease. These controversial results in early and late sepsis could provide one explanation for the contradictory conclusions of several clinical studies evaluating the role and prevalence of bacterial translocation in multi-organ failure and other syndromes.

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