Early events after intra-abdominal infection with
Bacteroides fragilis and Escherichia coli

W. R. VERWEIJ, F. NAMAVAR, W. F. SCHOUTEN and D. M. MACLAREN

Research Group for Commensal Infections, Department of Medical Microbiology, School of Medicine, Vrije Universiteit, v.d. Boechorststraat 7, P.O.B. 7161, 1007 MC Amsterdam, The Netherlands

Summary. Growth of Bacteroides fragilis and Escherichia coli was monitored during early stages of single (mono-) and mixed intra-abdominal infection in a rat fibrin clot model. When B. fragilis and E. coli were together involved in the infection, B. fragilis numbers increased about 6 h after an initial decline. This increase was not found with B. fragilis mono-infections. The numbers of E. coli increased rapidly in both mono- and mixed infections and stayed high for several days, but only mixed infection resulted in abscesses that persisted for more than 7 days. Macrophages, the main component of the peritoneal cellular defence mechanism, were outnumbered by polymorphonuclear leucocytes during the first 6 h of infection. Further characterisation of the macrophage population by means of monoclonal antibodies showed a shift from resident to exudate macrophages as the result of influx of the latter.

Introduction

Bacteroides fragilis and Escherichia coli are common pathogens in intra-abdominal infections. The contribution of each to the pathogenesis of mixed infections has been well demonstrated, particularly in animal models of intra-abdominal and subcutaneous infections. On the basis of several experimental animal studies, it has been postulated that these organisms act synergically, leading to either enhanced mortality or increased abscess formation. In these experimental studies, abscesses developed after 3–7 days. However, there is little information on the kinetics of bacterial growth during the early stages of infection. It is well known that free bacteria are readily removed from the peritoneal cavity into the bloodstream by the host peritoneal translymphatic system. Therefore, we studied the growth kinetics of the bacteria and the reaction of the cellular components of the peritoneal defences at early stages of infection. A rat fibrin clot model with some modifications was used. This model is analogous to the clinical situation in which bacteria spill into the peritoneal cavity after visceral perforation and are rapidly walled off by fibrinous exudate, mobile viscera and omentum.

Material and methods

Bacterial strains

The B. fragilis (BE1) and E. coli (EB1) strains studied had been isolated from the wound infection of a patient at the Academic Hospital of the Vrije Universiteit. They had been used in earlier studies.

Techniques of storage, growth conditions, ingredients of the minimal growth medium and method of enumeration have been previously described. For inoculation into the fibrin clot, B. fragilis and E. coli cultures (16–18 h) were pelleted by centrifugation, washed and resuspended in Hanks's Buffered Salts Solution (HBSS, Gibco) supplemented with cysteine hydrochloride 0.5 mg/L, and saline 0.9% solution. The concentrations of the bacterial suspensions were adjusted to 1 x 10^9 cfu/ml for B. fragilis and 1 x 10^6 cfu/ml for E. coli.

Incorporation of bacteria into fibrin clots

Infected fibrin clots were made by a modification of a previously described method. Briefly, human fibrinogen (Sigma) was suspended in calcium-free phosphate-buffered saline (PBS) to a concentration of 2 mg/ml and sterilised by ultraviolet irradiation for 10 min; 1.5-ml volumes were frozen at -80°C and thawed on ice before use. Thrombin (Sigma) was suspended in sterile distilled water to a concentration of 2 mg/ml and sterilised by ultraviolet irradiation for 10 min; 1-5-ml volumes were frozen at -80°C and thawed on ice before use. Thrombin (Sigma) was suspended in sterile distilled water to a concentration of 30 NIH units/ml and 250-μl samples were kept at -80°C. For the preparation of infected clots, 1.4 ml of fibrinogen was placed in a polystyrene tube and 100 μl of either B. fragilis or E. coli suspension, or both, were added. In monomicrobial clots, the appropriate control was used to replace the synergic bacterial partner. After gently mixing the tube, 200 μl of the thrombin solution was added and the tubes were gently mixed again. Fibrin clots formed almost
intra-peritoneal cell population were made from cytocentrifuge preparations (Shandon Southern Instruments Ltd, UK) stained with May-Grunwald Giemsa in which 500 cells were counted.

Further characterisation of the macrophage population was done with monoclonal antibodies (MAbs) ED1 and ED2 against rat macrophages, kindly provided by Dr C. D. Dijkstra (Department of Cell biology, Faculty of Medicine, Vrije Universiteit Amsterdam, The Netherlands). MAb ED1 has been shown to react with the total monocyte population and MAb ED2 with more than 50% of the resident macrophage population of the peritoneal cavity.\textsuperscript{15, 16} Macrophages expressing antigens recognised by MAbs ED1 and ED2 were detected by an immunoperoxidase procedure in cytospin centrifuge preparations with diaminobenzidine-tetra-HCl (DAB) as chromogen. Briefly, cytospin preparations, dried overnight on silica gel, were fixed in formalin 1% v/v in acetone, washed with PBS and incubated for 30–60 min with MAbs diluted in PBS supplemented with BSA 0.2% w/v. After washing three times with PBS, samples were incubated with rabbit anti-mouse IgG peroxidase procedure in cytospin centrifuge preparations with diaminobenzidine-tetra-HCl (DAB) as chromogen. Briefly, cytospin preparations, dried overnight on silica gel, were fixed in formalin 1% v/v in acetone, washed with PBS and incubated for 30–60 min with MAbs diluted in PBS supplemented with BSA 0.2% w/v. After washing three times with PBS, samples were incubated with rabbit anti-mouse IgG horseradish peroxidase (DAKO) diluted in PBS with BSA 0.2% and normal rat serum 1% for 30 min. Samples were washed again three times and incubated for 15 min with the chromogen DAB (0.5 mg/ml in 0.05 M Tris-HCl, pH 7.6) and H$_2$O$_2$ 0.03% v/v. After another three wash steps, the samples were counterstained with haematoxylin for 1 min. Sometimes an additional incubation in CuSO$_4$ 0.5% was used to "amplify" the staining. At least 400 cells were examined for the presence of the different antigens.

Results

Counts of bacteria in mono-infected fibrin clots

To obtain information about the early events after intra-abdominal infection, the bacterial contents of both peritoneal cavity and fibrin clot were monitored at 2-h intervals during the first 8 h and 5–7 days after implantation of the clot. When clots were infected with $2 \times 10^5$ cfu of \textit{E. coli} EB1 alone, an increase to $(4–5) \times 10^7$ cfu/clot was found within the first 8 h. Thereafter, the numbers of \textit{E. coli} in the clots did not change during the first 5 days but after more than 7 days there were less than 50 cfu/clot. When the clot was infected with \textit{B. fragilis} BE1 alone, the bacteria (and clot) disappeared within 24 h (fig. 1). Mono-infections with \textit{E. coli} EB1 led to small peritoneal abscesses, which disappeared after 7 days. Mono-infections with \textit{B. fragilis} BE1 did not lead to the development of abscesses. Bacterial content of the peritoneal cavity was highest 2 h after implantation and then decreased rapidly. The total number of free bacteria never exceeded 1% of the clot inoculum.

Counts of bacteria in mixed infected fibrin clots

Mixed infected clots were investigated at the same time intervals as with mono-infections. An inoculum of $(1–2) \times 10^8$ cfu of \textit{B. fragilis} and $(1–2) \times 10^5$ cfu of \textit{E. coli} in fibrin clots resulted in a rapid increase in numbers of \textit{E. coli} (fig. 2). Numbers of \textit{E. coli} remained high even after 7 days. On the other hand, numbers of \textit{B. fragilis} declined during the first 4–6 h after implantation of the clot. After 8 h, the numbers of \textit{B. fragilis} had increased to $(0–5–1) \times 10^8$ cfu/clot. This decrease in the count of \textit{B. fragilis} followed by a rise during the...
first 8 h was found consistently for all mixed infections (fig. 2). Animals with mixed infected clots developed larger abscesses than when E. coli alone was used. As with mono-infected clots, free bacteria were detectable only in the peritoneal cavity in very low numbers during the first few hours after implantation.

Counts and characterisation of peritoneal cells

The absolute numbers of the cell types in the peritoneal cavity of rats under normal conditions \((t=0)\) and during experimental mixed infection are shown in the table (viability was always \(\geq 95\%\)). Polymorphonuclear leucocytes (PMNL) were divided into eosinophils and other PMNL, mainly neutrophils. The remainder, designated the rest-group, consisted of mast cells and lymphocytes. The number of PMNL increased rapidly after implantation of fibrin clots and reached values of \(>4 \times 10^7\) cells/peritoneal cavity. This influx was due to the presence of the bacteria as well as being a reaction to the surgical trauma of implantation of the fibrin clot, because the influx of PMNL was lower when sterile clots were used. The peritoneal cell population returned to normal within a week when sterile clots were used.

To characterise the rat peritoneal macrophage population further, two MAbs directed against rat macrophages were used. Resident macrophages are the predominant type of peritoneal macrophages without stimulation. Fig. 3 shows the results of macrophage characterisation with these two MAbs. The decrease in the absolute number of macrophages during the early stages of infection was due to a decrease in resident \((ED1^+ / ED2^+)\) macrophages possibly due in part to leakage from the wound. On the other hand, the exudate \((ED1^+ / ED2^-)\) macrophages increased and at 8 h after implantation compensated for the loss of resident macrophages. The absolute number of \((1-2) \times 10^7\) macrophages in the peritoneal cavity of the rats corresponds well with values reported by others.\(^{17}\)

Discussion

Several animal models have been used previously to study mixed aerobic-anaerobic infections.\(^{18}\) In this study the rat fibrin clot model was adopted because it seemed the most suitable model for the study of intra-abdominal infections. It mimics very well the clinical situation in which bacteria spilled into the peritoneal cavity after visceral perforation are rapidly walled off by a fibrinous exudate, mobile viscera and omentum. This model was used to study the first 8-h period of a synergic infection with E. coli and B. fragilis and the results were compared with those obtained with the murine subcutaneous model.\(^{5}\)

Other investigators, who used the fibrin clot model, focused on abscess formation and abscess size after 5–7 days.\(^{6}\) They showed that these two parameters

---

**Figure 1.** Bacterial growth kinetics after implantation of monomicrobial fibrin clots containing \((1-2) \times 10^6\) cfu of B. fragilis BE1 (□) or \((1-2) \times 10^5\) cfu of E. coli EB1 (▲).

**Figure 2.** Bacterial growth kinetics of B. fragilis (■) and E. coli (▲) after implantation of mixed infected fibrin clots containing \((1-2) \times 10^6\) cfu of B. fragilis BE1 and \((1-2) \times 10^5\) cfu of E. coli EB1. Values are expressed as means and SD of four independent experiments.
were influenced by bacterial inocula as well as bacterial strains used. We used *E. coli* and *B. fragilis* strains isolated from a wound infection. The inocula of (1-2) x 10^8 cfu of *B. fragilis* and (1-2) x 10^5 cfu of *E. coli* resulted in persistent abscesses only when used in combination.

While studying the bacterial growth kinetics within the fibrin clot we observed what we regard as the “decisive moment” in abscess formation, about 6 h after infection. In contrast to the situation with mono-infected *B. fragilis* clots, the number of *B. fragilis* in mixed clots began to increase from 6 h after implantation after an initial decline to about 10% of the starting inoculum. The presence of *E. coli*, or conditions created by its presence and metabolism, enabled *B. fragilis* to survive instead of being cleared from the peritoneal cavity. The effects of *E. coli* on local conditions could be changes of pH, reduction of the oxygen level or excretion of “nutrients” for *B. fragilis*, or they might involve interference with chemotaxis or peritoneal cavity. The effects of *E. coli* were influenced by bacterial inocula as well as bacterial strains used. We used *E. coli* and *B. fragilis* strains isolated from a wound infection. The inocula of (1-2) x 10^8 cfu of *B. fragilis* and (1-2) x 10^5 cfu of *E. coli* resulted in persistent abscesses only when used in combination.

In this fibrin clot model the growth kinetics of *E. coli* in mono- and mixed infected clots are very similar; irrespective of the presence of *B. fragilis*. The numbers of *E. coli* increased rapidly and stayed at a high level for several days. The effect of the presence of *B. fragilis* on *E. coli* was seen only after 5 days when mono-abscesses with *E. coli* disappeared whereas abscesses caused by the combination of bacteria persisted (figs. 1 and 2). With the subcutaneous model there was a dose-dependent, reciprocal requirement of *E. coli* and *B. fragilis* to avoid clearance early after the injection. The differences in time and dose at which effects of *B. fragilis* on *E. coli* were seen in the fibrin clot model might be explained by the protective barrier provided by the fibrin clot.

In the experimental approach described here, whether the mixed infection progressed to an abscess or not seemed to be determined during the very early stages of infection. The importance of the first hours of an infection with respect to abscess formation can also be deduced from other experiments in which tissue plasminogen activator (t-PA) was used. It was shown, with the same rat fibrin clot model, that intra-abdominal abscess formation could be completely prevented when t-PA was given i.p. during, or by means of open lavage within a very short period after, surgery. More recently it was shown that an increased concentration of t-PA (0-1 mg/ml) prevented abscess formation after a 6-h delay. A decisive period in the early stages of infection with regard to antibiotic prophylaxis has previously been postulated with a guinea-pig model.

A decisive moment or period leading to an increase of *B. fragilis* in mixed infected clots after 6 h in this study might be explained by a change in clot structure or by decreased peritoneal host defence in consequence of an altered peritoneal cell population. Firstly, clot contraction alone or in combination with host fibrin deposition could interfere with clot infiltration by host defence cells and bacterial killing. Secondly, the altered peritoneal population might be less effective with regard to fibrinolytic and phagocytic capacities.

To investigate the latter possibility, an inventory of the peritoneal cell population was made during the early stages of infection. The results (table and fig. 3) show changes in both the total number of peritoneal

**Table.** Peritoneal subpopulations after implantation of fibrin clots infected with *B. fragilis* and *E. coli*

<table>
<thead>
<tr>
<th>Peritoneal cells</th>
<th>Mean* cell count (x 10^4) and (range)† at time (h) after clot implantation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0-6</td>
</tr>
<tr>
<td></td>
<td>(20-30)</td>
</tr>
<tr>
<td>PMNL‡</td>
<td>0-0</td>
</tr>
<tr>
<td></td>
<td>(0)</td>
</tr>
<tr>
<td>Macrophages</td>
<td>1-8</td>
</tr>
<tr>
<td></td>
<td>(67-79)</td>
</tr>
<tr>
<td>Rest</td>
<td>0-1</td>
</tr>
<tr>
<td></td>
<td>(4-6)</td>
</tr>
<tr>
<td>Total</td>
<td>2-4</td>
</tr>
</tbody>
</table>

* Mean of four independent experiments.
† Ranges as percentages of the total population.
‡ PMNL minus eosinophils.
cells and their distribution in several subpopulations. An influx of PMNL was found as a response to both surgical trauma and the presence of bacteria in the peritoneal cavity. The influx of these cells, mainly neutrophils, is in agreement with the observations of others who used a bacterial containment chamber in mice and after an i.p. injection of $2 \times 10^8$ dead E. coli cells.

It was observed that macrophages, the most common cell type in the peritoneal cavity at the start of the infection, were outnumbered by PMNL within 2 h after implantation of the mixed infected clot. However, although the total number of macrophages did not change markedly, it was possible to detect a shift from one subpopulation to another by means of MAbs ED1 and ED2. The increasing contribution of exudate macrophages (ED1 + /ED2 −) (fig. 3) may have important consequences for bacterial clearance and abscess formation. Resident macrophages (ED1 + /ED2 +) are differentiated phagocytic cells adjusted to the peritoneal environment. Exudate macrophages, on the other hand, are fresh blood monocytes; though these have phagocytic activity, they are less differentiated. Separation and characterisation of these cell types combined with functional assays may provide more information on their role in abscess formation.

References