In-vitro activity of peritoneal cells from rats after intra-abdominal infection with *Bacteroides fragilis* and *Escherichia coli*

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Summary. Peritoneal cells from rats infected intraperitoneally with *Escherichia coli* and *Bacteroides fragilis*, alone or in combination were examined *in vitro*. Cells were harvested 6 h after implantation of fibrin clots infected with *E. coli* or *B. fragilis*, separately or containing both species, and assayed for their bactericidal capacities, chemiluminescence and production of cidal metabolites. Peritoneal cell populations from rats with implants of any of the infected clots showed similar distribution of different subpopulations. Bactericidal activity of peritoneal cells did not differ with the bacterial species used. Chemiluminescence values of peritoneal cells from rats with mono-infected *B. fragilis* or mixed-infected implanted clots, after stimulation with either particles or chemical stimuli, were significantly higher than those of rats with mono-infected *E. coli* or sterile clots. The same tendency was seen with regard to the production of cidal metabolites such as hydrogen peroxide and superoxide anions although no significant differences were found.

Introduction

Intra-abdominal abscess formation after peritoneal infection is considered to be the result of both bacterial sequestration and cellular factors. Fibrinous exudate together with thrombin forms fibrinous and ultimately fibrous adhesions. These are capable of capturing bacteria that are not cleared by the lymphatic system or killed by the indigenous macrophages and recruited leucocytes. Subsequent infiltration of these infected fibrin matrices by leucocytes and secretion of proteolytic enzymes result in a circumscribed collection of pus. However, abscess formation is more than the result of an inefficient or overloaded clearing and killing mechanism.¹ In animal models of abscess formation it was shown that monomicrobial abscesses could be established only when either an extremely high inoculum was used or the strains were capsulate.^{2–4} Specific combinations of facultatively and obligately anaerobic bacteria, e.g., *Escherichia coli* and *Bacteroides fragilis*, on the other hand often resulted in abscess formation when clinically “significant” inocula were used, whether combined with adjuvant or not.^{5–8} In the case of the fibrin clot model, originally described by Ahrenholz and Simmons,⁹ the only “adjuvant” required with *E. coli* and *B. fragilis* was fibrinogen.^{2–10}

In the present study this rat fibrin clot model was used to produce intra-abdominal abscesses. Previous work had shown that persistent abscesses formed when both *E. coli* and *B. fragilis* were present,¹⁰ but, individually, either *B. fragilis* or *E. coli* was cleared from the peritoneal cavity within 48 h and 2 weeks respectively. We defined a decisive moment in abscess formation 6 h after implantation of a mixed-infected fibrin clot.¹⁰ This point was marked by an increase in the viable counts of *B. fragilis* in the mixed-infected clot after an initial decrease. In the mono-infected *B. fragilis* clot viable counts decreased steadily and both bacteria and clot were removed within 24 h. The present study was designed to investigate the cellular defence components of the peritoneum during the early stages of intra-abdominal mono- and mixed infection, and to determine whether the different sorts of infection had specific effects on cellular defence mechanisms. Cells of the peritoneal cavity were harvested 6 h after the implantation of mono- or mixed-infected fibrin clots. In-vitro assays were used to investigate bactericidal activity and production of cidal metabolites by the isolated cells.

Materials and methods

Bacterial strains

B. fragilis strain BE1 and *E. coli* strain EB1 are

clinical isolates and have been used in previous studies.¹⁰⁻¹² Techniques of storage, growth conditions and ingredients of the minimal growth media have been described previously.^{10,13} Bacterial cultures (16-h) were pelleted by centrifugation, washed and resuspended in Hanks's Balanced Salts Solution (HBSS, Gibco) supplemented with cysteine hydrochloride 0.5 mg/L for *B. fragilis*, and buffered saline (NaCl 0.9%, pH 7.4), for *E. coli* "clot suspensions" and HBSS for "killing suspensions". The bacterial concentrations of the suspensions were adjusted to $(1-2) \times 10^9$ cfu/ml for *B. fragilis* and $(1-2) \times 10^6$ cfu/ml for *E. coli*; 5×10^8 cfu/ml of the bacterial suspensions were used in killing studies.

Incorporation of bacteria into the fibrin clot

Sterile and infected fibrin clots were prepared as described previously.¹⁰ Briefly, 100- μ l samples of the bacterial suspensions or the appropriate buffer were added to 1.4 ml of human fibrinogen (Sigma) 2 mg/ml. After mixing the tube contents gently, 200 μ l of thrombin (30 NIH units/ml; Sigma) was added to catalyse fibrin clot formation.

Animals

Male Wistar rats (Harlan CPB, Zeist, The Netherlands) weighing 200 g on arrival were kept for a week before operation. During this period food and water were provided *ad libitum*. Fibrin clots, either sterile, mono- or mixed-infected, were implanted intraperitoneally (i.p.) via a 2-3-cm midline incision under ether anaesthesia. The incision was closed in two layers with four sutures each. Total anaesthesia time was < 10 min.¹⁰

Preparation and characterisation of the peritoneal cell population

The animals were killed by CO₂ 6 h after implantation of the fibrin clot. Peritoneal cells were harvested by lavage with 5 ml of buffered saline supplemented with bovine serum albumin (BSA) 0.2%. Differential cell counts were made from cytocentrifuge preparations (Shandon Southern Instruments Ltd, 93-96 Chadwick Road, Runcorn, Cheshire) stained with May-Grünwald Giemsa in which at least 500 cells were counted.

Measurement of killing

Peritoneal cell suspensions were washed and resuspended in HBSS with gelatin 0.1% and adjusted to a concentration of 1×10^7 cells/ml. Viability of the cells was determined by trypan blue exclusion. Reaction mixtures were prepared in sterile polypropylene tubes (12 $\times$ 75 mm, Greiner Labortechnik Ltd, Station Road, Cam. Dursley, Glos) each containing 0.5 ml peritoneal cell suspension, 0.1 ml of normal rat serum

(NRS), 0.1 ml of *E. coli* suspension (5×10^8 cfu/ml in HBSS) and 0.3 ml of HBSS with gelatin 0.1%. Tubes were incubated aerobically at 37°C in an IKA Vibrofix shaking apparatus (Rofa Mavi, The Netherlands) at 400 rpm. After 30, 60, 90, 120 and 150 min, 100- μ l samples were suspended in 9.9 ml of ice-cold distilled water and viable counts were performed by plating serial 10-fold dilutions on nutrient-agar plates.

Chemiluminescence (CL)

The reaction mixture for CL consisted of 0.1 ml of peritoneal cell suspension (5×10^6 cells/ml in HBSS without phenol red), 0.1 ml of 0.1 mM luminol (Lumac, Switzerland), 0.2 ml of phosphate-buffered saline (PBS) and 0.1 ml of 80 mM phorbol 12-myristate 13-acetate (PMA, Sigma). Samples were incubated at 37°C in a shaking water bath. CL was measured at 37°C in a Lumacounter model 2080 (Lumac) in the integral mode with a preset of 10 s. The mean of counts during five 2-s periods was recorded. Peak CL values were expressed as a percentage of the peak value found with cells from rats in which a sterile fibrin clot was implanted.

Measurement of hydrogen peroxide (H₂O₂) release from peritoneal cells

The release of H₂O₂ by the peritoneal cells was measured in a discontinuous phenol red oxidation assay.¹⁴ Briefly, wells of a 24-well tissue-culture plate (Falcon, USA) were filled with 0.5 ml of 56 mM phenol red solution in a buffer consisting of 140 mM NaCl, 10 mM phosphate buffer, pH 7.0, and 5.5 mM dextrose. To this solution, 50 μ l of horseradish peroxidase (200 U/ml, Sigma) and 50 μ l of 0.32 mM PMA was added. Finally 0.5 ml of peritoneal cell suspension (1×10^7 cells/ml in buffered saline) was added and plates were incubated aerobically at 37°C in a gently shaking water bath. After 30, 60 and 120 min, 1-ml samples were transferred to reaction vials containing 100 μ l of 0.2 M NaOH. Samples were then centrifuged briefly and the optical density of the supernate was measured at 540 nm (OD₅₄₀). Absolute amounts of H₂O₂ were calculated from a H₂O₂ standard curve.

Measurement of superoxide anion (O₂⁻) release by peritoneal cells

Release of O₂⁻ by peritoneal cells was measured with a discontinuous ferricytochrome C reduction assay.¹⁵ Briefly, wells of a 24-well tissue culture plate (Falcon, USA) were filled with 0.5 ml of 100 μ M cytochrome C solution (Boehringer, Mannheim, Germany). To this solution, 50 μ l of catalase (1 mg/ml, Sigma) and 50 μ l of 0.32 mM PMA were added. Finally 0.5 ml of peritoneal cell suspension (1×10^7 cells/ml in buffered saline) was added. Plates were incubated aerobically at 37°C in a gently shaking water bath. After 30, 60 and 120 min, 1-ml samples were trans-

Table I. Peritoneal subpopulations after implantation of infected and sterile fibrin clots

Bacteria	cfu†	Mean* percentage (SD) of cell subpopulations and total cell number 6 h after clot implantation				
		eosinophils	PMNL‡	macrophages	other cells§	Total
<i>B. fragilis</i> and <i>E. coli</i>	$(1-2) \times 10^8$	3 (2)	68 (5)	29 (4)	0 (1)	6.9×10^7
<i>E. coli</i>	$(1-2) \times 10^5$	4 (2)	61 (4)	34 (4)	1 (1)	5.2×10^7
<i>B. fragilis</i>	$(1-2) \times 10^5$	2 (1)	72 (4)	26 (3)	0 (1)	7.2×10^7
None (sterile)	$(1-2) \times 10^8$	6 (2)	51 (8)	41 (6)	2 (1)	5.0×10^7

* Mean of five independent experiments.

† Cfus inoculated in fibrin clot.

‡ PMNL minus eosinophils.

§ Mast cells and lymphocytes.

|| Total number of cells present in the rat peritoneal cavity (mean of five independent experiments).

ferred to reaction vials. Samples were then centrifuged briefly and the OD_{540} of the supernate was measured. Absolute amounts of $O_2^{\cdot-}$ were calculated from the millimolar extinction coefficient of ferrocyclochrome C (Fe^{2+}) of 21.1 at neutral pH.

Statistical analysis

Analysis of variance (ANOVA) for a two-factor design with randomised blocks was applied to the CL peak values of five independent experiments which were done either singly or multiply.¹⁶ Regression analysis, after log transformation, yielded the 95% confidence intervals for the ratio of the detected contrasts. Results from all other tests were analysed by Student's *t* test.

Results

Peritoneal cell population

Peritoneal cell populations of rats with mono-infected *E. coli* or *B. fragilis*, or mixed-infected fibrin clot implants were harvested 6 h after implantation of the clots and differentiated. Polymorphonuclear leucocytes (PMNL) were divided into eosinophils and other PMNL, mainly neutrophils. The remainder consisted of mast cells and lymphocytes.

Table I shows the percentages of the different cell types present 6 h after implantation of the fibrin clot. These data show that at the early stage of infection the composition of the peritoneal cell population of rats with infected implanted clots was very similar. Sterile clots, however, induced a smaller influx of peripheral blood cells resulting in a higher percentage of macrophages and eosinophils.

Killing studies

To determine whether the mono- and mixed infections had different influences on the capacities of peritoneal cells present 6 h after implantation of the fibrin clots, these cells were tested in a killing assay that detects the removal of bacteria by phagocytosis and

Figure. Killing of *E. coli* EB1 by peritoneal cells from rats, 6 h after implantation of: sterile fibrin clot (x); *E. coli*-infected fibrin clot (O); *B. fragilis*-infected fibrin clot (□); double-infected fibrin clot (△); growth control (◇).

subsequent killing by the peritoneal cells in the presence of NRS 10%. The bacteria: phagocyte ratio was 10:1.

The figure shows the viable counts of *E. coli* EB1 after incubation with peritoneal cells from rats with sterile, mono- or mixed-infected *E. coli* or *B. fragilis* fibrin clot implants. A reduction of > 90% in viable counts of *E. coli* was observed after 90 min. *E. coli* viability was not affected by serum alone. No significant differences were found in the bactericidal capacity of peritoneal cells isolated 6 h after implantation of

Table II. CL peak values of rat peritoneal cells isolated 6 h after implantation of the fibrin clot

Bacteria present in clot	cfu*	Percentage relative CL chemiluminescence† (SEM)	p value
<i>B. fragilis</i> and <i>E. coli</i>	$(1-2) \times 10^8$	198 (59)	†
<i>E. coli</i>	$(1-2) \times 10^5$	131 (38)	0.1096§
<i>B. fragilis</i>	$(1-2) \times 10^8$	173 (56)	0.0016§
None (sterile)		100	

* CfU inoculated in fibrin clot.

† Maximum CL values expressed as percentage of maximum values with peritoneal cells from rats with sterile fibrin clot implants. Values are expressed as mean (SEM) of five experiments.

‡ P value of effect modification = 0.6573 (interaction between *E. coli* and *B. fragilis*).

§ P value pertaining to main effects in two-way ANOVA.

mono-infected *E. coli* or *B. fragilis* clots or mixed-infected clots.

Chemiluminescence

CL of the peritoneal cells was measured after stimulation with zymosan, bacteria and PMA. Whereas the absolute CL value differed between each stimulus, cells giving the highest CL peak did so with all stimuli.

Table II shows the peak CL values, after stimulation with PMA, expressed as percentages of the maximum values found with cells from rats with sterile fibrin clot implants. Analysis of the data by the two-factor design yielded a 95% confidence interval for *E. coli* in the presence versus absence of *B. fragilis* of 95–154%, $p = 0.110$. For *B. fragilis* these values were 123–198%, $p = 0.002$, indicating significantly elevated levels of CL due to the presence of *B. fragilis* in the infected clot. CL values of cells from rats with mixed-infected clot implants were also significantly elevated compared to those from rats with sterile clots. No statistical significance was found when comparing *E. coli* and *B. fragilis* ($p = 0.657$).

H₂O₂ and O₂^{•-} production

The amounts of H₂O₂ and O₂^{•-} produced by 10⁶ peritoneal cells after 30, 60 and 120 min are shown in

table III. Peritoneal cells from rats with sterile fibrin clots produced only small amounts of H₂O₂. No significant differences were found in the production of this metabolite by cells from rats with mono- or mixed-infected clots. Production of O₂^{•-} was extremely low in all samples.

Discussion

In most studies, the effects of bacteria and bacterial products on host defences have been investigated with human peripheral blood PMNL.^{11,12,17} However, in this study we were interested in the defence capacities of host cells present in the peritoneal cavity during the early stages of intra-abdominal infection. Therefore, we used an animal model in which infected fibrin clots were implanted intraperitoneally in rats. The two bacterial strains used in this study, *B. fragilis* BE1 and *E. coli* EB1, lead to persistent abscess formation only when used in combination. This might result from a suppression of local host defences in mixed *E. coli* and *B. fragilis* infections. Therefore, we compared the efficacy of bacterial killing, CL and production of H₂O₂ and O₂^{•-} by peritoneal cells from rats with mono- or mixed-infected fibrin clot implants. Cells were harvested from rats 6 h after implantation of the fibrin clot, since previous studies had shown that this was a decisive moment in abscess formation.¹⁰ Bacterial killing, under the assay conditions used *in vitro*, was efficient and proved to be independent of the bacterial species present in the implanted fibrin clot. Other investigators have shown that bacterial killing by human PMNL was markedly reduced on incubation with *B. fragilis* culture filtrate or short chain fatty acids (SCFA) such as succinate at low pH.^{12,17} Rotstein *et al.*¹⁷ suggested that the co-existence of SCFA, produced by *B. fragilis*, and a low pH may account for increased survival rates in mixed infection. These findings may have implications *in vivo* since Bryant *et al.* reported that pH values of human pus sometimes drop below 6.0.¹⁸ However, other investigators have found that intraperitoneal pH changes during infection are only minor and never fall much below pH 6.¹⁹ Furthermore, there are no data on pH values or succinate concentrations within fibrin deposits during the early stages of infection. In this

Table III. Production of H₂O₂ and O₂^{•-} by rat peritoneal cells isolated 6 h after implantation of the fibrin clot

Bacteria present in clot	cfu‡	Mean nmol (SEM)* of H ₂ O ₂ produced after			Mean nmol (SEM)* of O ₂ ^{•-} produced after		
		30 min	60 min	120 min	30 min	60 min	120 min
<i>B. fragilis</i> and <i>E. coli</i>	$(1-2) \times 10^8$	7.4 (2.7)	12.2 (4.0)	15.7 (3.0)	2.6 (1.4)	3.7 (0.8)	4.2 (0.3)
<i>E. coli</i>	$(1-2) \times 10^5$	6.0 (3.0)	8.4 (5.6)	10.4 (6.0)	2.0 (0.2)	2.9 (1.1)	3.3 (2.0)
<i>B. fragilis</i>	$(1-2) \times 10^8$	6.7 (3.4)	11.2 (4.2)	14.1 (2.1)	2.5 (0.4)	3.8 (0.9)	4.5 (1.3)
None (sterile)		4.8 (1.2)	7.9 (1.2)	9.5 (0.20)	2.7 (1.1)	2.8 (1.3)	2.7 (1.1)

* Values are expressed as mean (SEM) of two experiments.

‡ CfU inoculated in fibrin clot.

study, CL showed the highest response by peritoneal cells from rats with mono-infected *B. fragilis* and mixed-infected fibrin clot implants. This effect might be accounted for by the presence of *B. fragilis* itself or by metabolites produced during the first 6 h. We excluded the possibility that these results were due only to a difference in priming of the peritoneal cells by a different number of particles, by testing cells from a rat with a fibrin clot implant containing $(1-2) \times 10^8$ cfu of *E. coli*. CL values of these cells were only slightly higher than those of rats with a fibrin clot implant containing $(1-2) \times 10^5$ cfu, indicating that priming by greater numbers of micro-organisms does not account for the observed difference.

The production of H_2O_2 and $O_2^{\cdot-}$ by rat peritoneal cells involved in bacterial killing was also studied. The production of H_2O_2 by these cells proved to be independent of the bacterial species in the implanted fibrin clot. Priming of the peritoneal cells *in vivo* by the presence of bacteria induced a two-fold increase in response compared to the control (sterile clot), but no significant differences were found between cells from rats with mono-infections of *E. coli* or *B. fragilis* or mixed-infected clots. $O_2^{\cdot-}$ production by rat peritoneal cells, as measured by ferricytochrome C reduction, was very low. Other investigators have demonstrated that H_2O_2 and $O_2^{\cdot-}$ production by human PMNL *in vitro* was significantly reduced after incubation with several SCFA at pH 5.5.²⁰ This phenomenon was seen on stimulation with opsonised zymosan or PMA and proved irreversible. In our studies, peritoneal cells from rats with fibrin clot implants did not show this phenomenon. This may

indicate that local intraperitoneal conditions are not comparable with the assay conditions *in vitro*, or may reflect differences between human peripheral blood PMNL and rat peritoneal cells.

Investigators who used a mouse model of intra-abdominal infection have looked at possible differences between abscess-derived and peritoneal exudate neutrophils.²¹⁻²³ At first no differences were detected in functional properties of the cells such as phagocytosis of *P. mirabilis*, CR3 expression and H_2O_2 production. However, analysis by flow cytometry of the respiratory burst after stimulation with bacteria in the presence of normal mouse serum revealed a variable, poorly responding subpopulation of neutrophils. The cellular defect causing this remains unknown.

The overall results of this study show that rat peritoneal cells isolated 6 h after implantation of mono- or mixed-infected fibrin clots demonstrated similar cellular activity. CL is the only assay that gave a clear distinction between cells from rats with sterile clots and those with monomicrobial or polymicrobial infection, suggesting involvement of myeloperoxidase-dependent singlet oxygen. This could mean that the findings of abscess formation *in vivo* in the case of mixed infection fibrin clots are probably not due merely to altered defence capacities of the peritoneal cell population. However, local soluble factors of either bacterial or host origin, present in the peritoneum during the early stages of infection, could influence cellular defence. We are currently studying the role of soluble factors on host defence in this rat model after implantation of mono- and mixed-infected fibrin clots.

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