Migration of rat peritoneal cells after intra-abdominal infection with *Bacteroides fragilis* and *Escherichia coli*

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A fibrin clot model for intra-abdominal abscess formation was used to study the migratory properties of peritoneal cells from rats during the early stages of infection. Peritoneal cells and fibrin clot remnant were harvested 6 h after implantation of a sterile, singly infected (*Escherichia coli* or *Bacteroides fragilis*) or mixed infected (*E. coli* and *B. fragilis*) fibrin clot. Histological study of fibrin clots, removed 6 h after implantation, showed a deeper infiltration by host cells of *B. fragilis* infected clots compared to the others. This difference in infiltration by peritoneal cells was not due to differences in fibrinolytic activity of the bacterial strains. Differential cell counts of the peritoneal cells from rats implanted with sterile, singly and mixed infected fibrin clots showed distribution over subpopulations to be independent of the bacterial content of the infected clots used. *In vitro* migration assays showed no significant differences in migration by peritoneal cells from rats implanted with clots containing a different bacterial composition. Since *B. fragilis* infected fibrin clots were more deeply infiltrated by host defence cells than the other clots, and only mixed infected clots led to persistent abscesses in this model, we conclude that local conditions within the fibrin matrix rather than intrinsic cellular capacities of the host cells are important for the process of abscess formation.

Introduction

Intra-abdominal abscesses are a result of an inflammatory response in which polymorphonuclear leucocytes (PMNL) play a key role. The formation of fibrous adhesions containing bacteria and the failure of the lymphatic system, indigenous macrophages and recruited PMNL to cope with the contamination, eventually leads to the formation of a circumscribed collection of pus. In our previous study we focused on the phagocytic and killing capacities of host cells during the early stage of mixed intra-abdominal infection (Verweij *et al.*, 1993). The ability of leucocytes to migrate towards and into an injured or contaminated site is another essential step in the process of abscess formation. Studies of chemotaxis and random migration of host cells in response to bacterial infection frequently use *in vitro* assays and peripheral human leucocytes from healthy donors. Using this approach an inhibition of chemotaxis by bacterial products was observed (Rotstein *et al.*, 1985, 1986;

Namavar *et al.*, 1987). Solomkin *et al.* (1981) used PMNL from patients with intra-abdominal infection and showed that these cells were depressed in their chemotaxis and spontaneous migration. In the study presented here we investigated *in vivo* and *in vitro* migratory abilities of the peritoneal cell population actually present during the early stages of single and mixed *Escherichia coli*/*Bacteroides fragilis* infections. We used a rat fibrin clot model for intra-abdominal abscess formation, which in previous studies led only to persistent abscesses when both bacterial species were present (Verweij *et al.*, 1991).

In our previous studies, using this model, we defined a decisive moment in abscess formation, 6 h after clot implantation. This point was marked by an increase in viable counts of *B. fragilis* in the mixed infected clot after an initial decrease, whereas in the *B. fragilis* singly infected clot there was a clear decrease. In the latter case both bacteria and fibrin clot were removed by host defences within 24 h, in contrast to the persistent abscesses that resulted from mixed infected clots. We therefore focused our attention on 6 h after clot implantation when studying migratory abilities of peritoneal cells. This was done by determining the infiltration zone of the clot remnant by host cells and the *in vitro*

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Abbreviations: FCS, foetal calf serum; mAb, monoclonal antibody; PMNL, polymorphonuclear leucocytes.

capacity of peritoneal cells to migrate towards a synthetic chemoattractant.

We also studied the possibility of fibrinolytic activity by *B. fragilis* and *E. coli*. This activity might have led to degradation of the fibrin clot matrix and thereby eased infiltration by host cells.

Methods

Bacterial strains. Both the *Bacteroides fragilis* (BE1) and the *Escherichia coli* (EB1), serotype O8:K43 strain used in the present study are clinical isolates and have been used in previous studies (Verweij *et al.*, 1991; Otto *et al.*, 1988; Namavar *et al.*, 1987; Verweij-Van Vught *et al.*, 1986). Techniques of storage, growth conditions and ingredients of the minimal growth medium (MM-medium) have been described previously (Namavar *et al.*, 1983). For inoculation of the fibrin clots and killing experiments late-exponential (16 h) cultures were pelleted by centrifugation, washed and resuspended in Hank's Buffered Salts solution (HBSS, Gibco) supplemented with 0.5 mg⁻¹ cysteine hydrochloride l⁻¹ for *B. fragilis* and buffered saline (0.9%, pH 7.4) for *E. coli*. The bacterial concentrations of the suspensions were adjusted to 1–2 × 10⁹ c.f.u. ml⁻¹ for *B. fragilis* and 1–2 × 10⁶ c.f.u. ml⁻¹ for *E. coli*. For measurement of fibrinolytic activity 50% (v/v) foetal calf serum (FCS) diluted 1:1 with PBS (137 mM-NaCl, 2.68 mM-KCl, 6.46 mM-Na₂HPO₄·2H₂O, 1.47 mM-KH₂PO₄) was used together with the standard media for culturing EB1 and BE1. A fresh isolate of *Enterococcus faecalis* as cultured on Lab-Lemco Broth (Oxoid) and MM-medium and *Porphyromonas gingivalis* HG 66 (= W83, H. N. Shah) in phosphate-buffered basic medium (BMP; Verweij-Van Vught *et al.*, 1986) supplemented with haemin (5 mg l⁻¹) and menadione (2 mg l⁻¹) (h/m).

Incorporation of bacteria into the fibrin clot. Sterile and bacterially infected fibrin clots were made as previously described (Verweij *et al.*, 1991). Briefly, 100 µl of the bacterial suspensions or the appropriate buffer were added to 1.4 ml of human fibrinogen (Sigma; 2 mg ml⁻¹ in calcium- and magnesium-free PBS). After gently mixing the tubes, 200 µl thrombin (Sigma; 30 NIH units ml⁻¹ in sterile distilled water) was added to catalyse fibrin clot formation. Both the harvesting of bacteria and the incorporation into the fibrin clot were performed under normal atmospheric conditions, maximal time between cell harvesting and implantation of the fibrin clots was 2.5 h.

Animals and surgical procedure. Male Wistar rats (Harlan CPB, Zeist, The Netherlands) weighing 200 g on arrival were allowed to adapt for a week before operation. During this period food and water were provided *ad libitum*. Fibrin clots, either sterile, singly or mixed infected ones, were implanted intra-peritoneally (i.p.) via a 2–3 cm midline incision made under ether anaesthesia. The incision was closed in two layers with four sutures each. Total anaesthesia time was less than 10 min (Verweij *et al.*, 1991).

Preparation and characterization of the peritoneal cell population. The animals were killed by inhalation of CO₂ 6 h after implantation of the fibrin clot. Peritoneal cells were harvested by lavage with 5 ml buffered saline supplemented with 0.2% bovine serum albumin (BSA) and kept on ice until used. After complete opening of the peritoneal cavity the remnant of the fibrin clot was taken out, weighed and either frozen in liquid N₂ or washed once and treated with 1 ml trypsin (Sigma; 1000 U ml⁻¹ in 50 mM-Tris/HCl, pH 7.3). Peritoneal cell samples were counted in a Bürker chamber and viability was determined by trypan blue exclusion. The total numbers of cells were calculated by correcting for the volume that could be withdrawn from the peritoneal cavity. Differential cell counts were made from cytocentrifuge preparations

(Shandon Southern Instruments Ltd) stained with May–Grünwald Giemsa in which 500 cells were counted.

Fibrin clot infiltration. For histological assessment 8–12 µm sections of the frozen fibrin clots were air-dried and stored overnight on silica gel. Fibrin clot sections were stained with May–Grünwald and Gram stain and infiltration zones of the peritoneal cells were scored semi-quantitatively.

Further characterization of the infiltrating cell population was done with monoclonal antibody (mAb) ED1 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 (Dijkstra *et al.*, 1985; Beelen *et al.*, 1987). Macrophages expressing the antigen recognized by mAb ED1 were detected by an immunoperoxidase procedure in ultrathin sections with 3,3'-diaminobenzidine-tetrahydrochloride (DAB) (Sigma) as chromogen. Briefly, preparations, dried overnight on silica gel, were fixed in 1% (v/v) formalin in acetone, washed with PBS and incubated for 30–60 min with mAb diluted in PBS supplemented with 0.2% (w/v) BSA. After being washed three times with PBS, samples were incubated with rabbit anti-mouse IgG horseradish peroxidase (DAKO) diluted in PBS with 0.2% BSA and 1% normal rat serum for 30 min. Samples were washed again three times and incubated for 15 min with the chromogen DAB (0.5 mg ml⁻¹ in 0.05 M-Tris/HCl, pH 7.6) and 0.03% (v/v) H₂O₂. After three wash steps the samples were counterstained with haematoxylin for 1 min. Sometimes an additional incubation in 0.5% CuSO₄ was used to 'amplify' the staining.

In vitro migration. The chemotaxis of the peritoneal cells was determined by chemotactic assay in agarose as previously described (Namavar *et al.*, 1984). The media contained 1.2% (w/v) agarose, 10 ml 10 × TC medium 199 (Difco), 1 ml 7.5% (w/v) sodium bicarbonate, 10 ml inactivated new-born calf serum and 79 ml distilled water. A 10 ml volume of the agarose medium was put into tissue culture plates (60 × 15 mm, Falcon). Four series of three wells, 3 mm in diameter and 3 mm apart, were punched in the agarose gel. The centre well of each three-well series received 10 µl peritoneal cell suspension (10⁷ cells ml⁻¹ in TC medium 199), the outer well received 10 µl 2 × 10⁻⁷ M-f-Met-Leu-Phe in PBS and the inner well control medium. The plates were incubated at 37 °C in a humidified atmosphere containing 5% (v/v) CO₂ in air. After 3 h of incubation the plates were flooded with 5 ml methanol (99%, v/v) for 30 min and then after decanting the methanol they were flooded with the same volume of 4% (v/v) formalin for another 30 min. The agarose was removed and cells stained with Giemsa stain. Migration was scored under a microscope (total magnification 25 ×) and chemotaxis was expressed as the difference between directed migration and random migration.

Statistical analysis of the data was performed using Student's *t*-test.

Fibrinolytic activity. Bovine fibrinogen (Sigma, 12 mg ml⁻¹) was dissolved in PBS without Ca²⁺ and Mg²⁺ (Gibco) and sterilized by UV irradiation for 30 min. Thrombin was suspended in sterile distilled water to a concentration of 30 NIH units ml⁻¹. Top layers were made by adding 200 µl thrombin to 2.8 ml fibrinogen and, after being gently mixed, were poured over blood-agar plates. Plates were allowed to stand for 30 min under UV irradiation and kept at 4 °C until used. Bacterial suspensions and culture supernatant (5 µl) were tested for fibrinolytic activity after 48 h aerobic or anaerobic incubation at 37 °C. Fibrinolytic activity was recorded as a clear zone around the inoculum spot. Results were expressed on a semi-quantitative scale.

Detection of capsules. Capsules were detected by negative staining with Indian ink. A drop of a 16 h broth culture (late exponential phase) was mixed with a drop of 10% (w/v) glucose and a drop of Indian ink on a microscope slide. This was spread over the slide, allowed to air-dry, fixed with ethanol and stained with ammonium crystal violet. Wet

Table 1. *Parameters of clot remnant and cellular host defence of the rat peritoneal cavity 6 h after implantation of the fibrin clot*

The implanted clots were inoculated with $1-2 \times 10^5$ c.f.u. *E. coli* and/or $1-2 \times 10^8$ c.f.u. *B. fragilis*. The results are means or means $\pm$ SD of three experiments except in the case of the clot mass which is the mean $\pm$ SD of six experiments.

Fibrin clot		Type of fibrin clot			
		Mixed	<i>E. coli</i>	<i>B. fragilis</i>	Sterile
c.f.u. present in clot	<i>E. coli</i>	$3-7 \times 10^7$	$1-3 \times 10^7$	—	—
	<i>B. fragilis</i>	$2-4 \times 10^7$	—	$2-3 \times 10^7$	—
Clot mass on isolation (g)		0.15 ± 0.03	0.21 ± 0.02	0.13 ± 0.02	0.24 ± 0.03
'Free' peritoneal cell population*	Total	6.9×10^7	5.2×10^7	7.2×10^7	5.0×10^7
	% PMNL	68 ± 5	61 ± 4	72 ± 4	51 ± 8
	% MO	29 ± 4	34 ± 4	26 ± 3	41 ± 6
	% EOS	3 ± 2	4 ± 2	2 ± 1	6 ± 2
	% Rest	0 ± 1	1 ± 1	0 ± 1	2 ± 1
Peritoneal cells present in fibrin clot	% PMNL	74 ± 4	67 ± 8	79 ± 4	55 ± 6
	% MO	24 ± 4	32 ± 8	19 ± 4	41 ± 6
	% EOS	2 ± 1	1 ± 1	2 ± 1	3 ± 2
	% Rest	0 ± 1	0 ± 1	0 ± 0	1 ± 1
Infiltration zone by host peritoneal cells†		+++	++	++++	+

* Total number of host defence cells in peritoneal cavity. PMNL, PMNL minus eosinophils; MO, macrophages; EOS, eosinophils; Rest, mast cells and lymphocytes.

† Light microscopical (semi-quantitative) infiltration score, verified by multiple experiments. +, minimal infiltration; ++, slight infiltration; +++, moderate infiltration; +++, deep infiltration.

suspensions were also made by mixing the bacterial suspension, glucose and ink on a slide, placing a coverslip on the mixture and blotting off the excess. The slides were examined by light microscopy.

Results

Peritoneal cell population

The percentages and absolute numbers of the cell types present in the peritoneal cavity at 6 h after implantation of the different kinds of fibrin clots are shown in Table 1. This Table also shows the percentages of the cell types present in the fibrin clots 6 h after implantation.

In bacterially infected clots the population of infiltrating cells seems a fair reflection of the 'free' cells in the peritoneum. The percentage of PMNL, however, is somewhat higher especially in the case of *B. fragilis* infected fibrin clots.

Fibrin clot infiltration by peritoneal cells

Figs 1(a-d) are light microscope photographs of May-Grünwald Giemsa-stained ultrathin sections of the fibrin clots which were removed 6 h after implantation. These photographs show the infiltration zone by host

defence cells of the fibrin matrix. As can be seen infiltration by host cells of sterile clot matrix (Fig. 1a) is minimal. Fibrin clots infected with a pure culture of *E. coli* showed a slight infiltration zone (Fig. 1b). A deeper zone of infiltration by host cells was seen in clots infected with a combination of *E. coli* and *B. fragilis* (Fig. 1c). The deepest infiltration zone was seen in the case of a single *B. fragilis* inoculum (Fig. 1d).

Further characterization of the cell population infiltrating the fibrin clot using mAb ED1, indicated that PMNL, predominantly neutrophils, penetrated deepest whereas macrophages were located as a layer on the periphery (results not shown).

In vitro migration

To find out whether differences in fibrin clot infiltration were due to different migratory capacities of peritoneal cells as a consequence of different bacterial inocula in the clots, peritoneal cells of rats implanted with sterile, singly, and mixed infected *B. fragilis* and *E. coli* fibrin clots were assayed *in vitro*. Table 2 shows the migratory capacity of the cells from rats implanted with different kinds of bacterial infected clots. These results show that

Fig. 1. May-Grünwald-stained ultrathin sections (12 µm) of fibrin clots removed 6 h after implantation. Clots infected with: (a), nothing (sterile clot); (b), *E. coli* ($1-2 \times 10^5$ c.f.u.); (c), *E. coli* ($1-2 \times 10^5$ c.f.u.) + *B. fragilis* ($1-2 \times 10^7$ c.f.u.) (mixed infected clot); (d), *B. fragilis* ($1-2 \times 10^7$ c.f.u.). cm, Clot matrix; iz, infiltration zone. Bars, 50 µm.

Table 2. *In vitro* migration of peritoneal cells towards a synthetic chemoattractant

Cells were isolated from rats 6 h after implantation of the fibrin clot. The chemoattractant was 2×10^{-7} M-f-Met-Leu-Phe. Values are expressed as mean $\pm$ SE of five experiments.

Bacteria present in fibrin clot	Chemotactic difference*
<i>B. fragilis</i> ($1-2 \times 10^8$)	
+ <i>E. coli</i> ($1-2 \times 10^5$)	1.5 ± 0.20
<i>E. coli</i> ($1-2 \times 10^5$)	1.8 ± 0.25
<i>B. fragilis</i> ($1-2 \times 10^8$)	1.6 ± 0.30
None (sterile clot)	1.3 ± 0.15

* Chemotactic difference expressed in mm as the difference between migration in response to f-Met-Leu-Phe and random migration.

the observed *in vivo* differences in clot infiltration are not reflected in significant differences in *in vitro* migration.

Fibrinolytic activity

To find out whether intrinsic fibrinolytic activity of the micro-organisms contributes to the differences in clot infiltration a fibrinolytic plate-assay was carried out. The results summarized in Table 3 show that neither

B. fragilis nor *E. coli* demonstrated any cell-bound or excreted fibrinolytic activity under the growth conditions tested. *P. gingivalis* HG 66 and *Ent. faecalis*, which were used as positive controls, gave clear zones of more than 5 and 10 mm in diameter respectively.

Capsule staining

Both the *B. fragilis* (BE1) and the *E. coli* (EB1) strains used, possessed a large capsule: small numbers of uncapsulated cells were seen in both air-dried and wet preparations (up to $\pm 5\%$ in *E. coli* preparations and $\pm 15\%$ in *B. fragilis* preparations).

Discussion

Several aspects of host defence during mixed intra-abdominal infection and abscess formation have been studied by different groups using various *in vivo* and *in vitro* models (Weinstein *et al.*, 1974; Hagen *et al.*, 1983; Nulsen *et al.*, 1983; Ahrenholz & Simmons, 1980; Onderdonk *et al.*, 1989). Special attention has been given to the phenomenon of pathogenic synergy by *B. fragilis* and *E. coli*. In this study we focused our attention on the

Table 3. Fibrinolytic activity in bacterial suspensions and culture supernatant (5 µl) after 48 h incubation at 37 °C in various media

Micro-organism (strain)	Lab-Lemco		MM medium		50 % FCS		BMP (+h/m)	
	Suspension	Supernatant	Suspension	Supernatant	Suspension	Supernatant	Suspension	Supernatant
<i>E. coli</i> (EB1)	—	—	—	—	—	—	ND	ND
<i>B. fragilis</i> (BE1)	ND	ND	—	—	—	—	—	—
<i>P. gingivalis</i> (HG 66)	ND	ND	ND	ND	ND	ND	+	+
<i>E. faecalis</i> *	++	++	++	++	ND	ND	ND	ND

—, No clear zone around application spot or diameter increase < 1 mm; +, increase of application zone diameter between 1 and 10 mm; ++, increase of application zone diameter > 10 mm. ND, Not determined.

* Fresh isolate.

early stages of infection, both mixed and single, and the capacity of host defence cells to migrate towards the site of infection.

We used an animal model in which bacterial infected fibrin clots were implanted i.p. in rats. Persistent abscesses only formed when both bacterial species were present whereas singly infected fibrin clots with *E. coli*, as the inoculum used, led to abscesses that were cleared within 10 d. *B. fragilis* alone was rapidly removed without abscess formation (Verweij *et al.*, 1991). Bacterial interference with recruitment and mobility of cellular host defence may explain these differences.

Results of *in vitro* studies on the effect of *E. coli* and *B. fragilis* on chemotaxis of human peripheral PMNL characterized *B. fragilis* as a poor inducer, even able to inhibit the chemotaxis of PMNL towards *E. coli* (Namavar *et al.*, 1984, 1987; Rotstein *et al.*, 1986). These data were partly explained by an inhibitory or even leucotoxic effect of short chain fatty acids, in particular by succinate, produced by *B. fragilis* (Rotstein *et al.*, 1985, 1986, 1987). The purulence of clinical mixed infections with *B. fragilis* and the profound infiltration by peritoneal cells of *B. fragilis* infected fibrin clots in this model which has a close resemblance to the clinical situation, throws doubts on the *in vivo* significance of these earlier findings.

The migration of peritoneal cells in the fibrin matrix during the first 6 h after implantation is much greater in the case of *B. fragilis* infected clots than *E. coli* or mixed infected clots. In the *in vivo* model these clots do not lead to abscess formation. The PMNL are clearly able to phagocytose and remove *B. fragilis* cells. When the infiltration zones of single *B. fragilis* and mixed infected clots are compared it appears that it is the presence of *E. coli* rather than *B. fragilis* that hampers peritoneal cell infiltration. This may have an important effect on abscess formation. Although *E. coli* alone leads to abscesses, our *in vivo* results show that *B. fragilis* is necessary for their persistence.

The greater infiltration and smaller weight of the clot

remnant at 6 h after implantation in the case of *B. fragilis* infected clots could also result from enzymic breakdown of the fibrin clot matrix leading to a more readily penetrated and open structure. Our fibrinolytic assay did not indicate that bacterial enzymes led to alterations in the fibrin matrix. Even under iron-limited conditions during growth in 50 % FCS, which are considered more appropriate to *in vivo* conditions, neither *B. fragilis* nor *E. coli* gave clear zones on 'fibrin-plates' whereas positive controls (*P. gingivalis* and *Ent. faecalis*) did.

Since migration towards a synthetic chemoattractant (f-Met-Leu-Phe) by peritoneal cells from rats implanted with sterile, singly or mixed infected clots did not differ significantly, it seems that the hampering of the migration of peritoneal defence cells seen *in vivo* resulted more from the local environment in the fibrin clot than from a modification of the chemotactic behaviour of the peritoneal cells.

Another possible explanation for the differences between *in vivo* and *in vitro* results could be that different receptors or processes are involved in chemotaxis towards micro-organisms and the synthetic chemoattractant f-Met-Leu-Phe so that there are indeed alterations in chemotactic functions brought about by different bacterial inocula; this seems less likely.

Our results are in contrast to the previous *in vitro* findings of others as well as of our own group, who found that *B. fragilis* and its metabolites such as succinate seemed to play a key role in frustrating host defence during mixed intra-abdominal infections (Namavar *et al.*, 1984, 1987; Rotstein *et al.*, 1985, 1986, 1987). However, in those studies human peripheral PMNL were used instead of the actual cell population present at the site of infection. The PMNL may very well respond differently from peritoneal cells that are adapted to their own environment. Secondly the *in vitro* culturing of *E. coli* and *B. fragilis* and the media used may influence the metabolic routes and thereby short chain fatty acid production and concentration in the supernatant. In the model we used, washed bacterial cells are implanted and

bacterial growth and metabolism in the fibrin clot, during the 6 h residence, takes place under *in vivo* conditions. Under these conditions an irreversible effect on the migration of free peritoneal cells by the fatty acids which might have been produced was not detected. However, it is not clear to what extent these cells reflect gut-derived bacteria. Study of bacteria cultured under simulated *in vivo* conditions, as regards pH and concentration of nutrients, iron and serum proteins is needed to answer this question.

Our study shows that in this model the role of *B. fragilis* in the initial phase of abscess formation is modest as is its effect on migration of peritoneal cells. It is the presence of *E. coli* that seems to hamper host defence, thereby permitting *B. fragilis* to establish itself. For the persistence of abscesses the presence of *B. fragilis* appears essential. The mechanisms by which *E. coli* interferes with host defence and favours the growth of *B. fragilis* in this model need further investigation.

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