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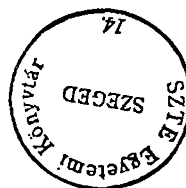
Examination of pathogenic factors of anaerobic bacteria

PhD Thesis

Ildikó Szőke

1999



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PhD Thesis

Ildikó Szőke

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References
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Communications connected with the thesis

I. I. Szőke, C. Pascu, E. Nagy, A. Ljung, T. Wadström
Binding of extracellular matrix proteins to the surface of anaerobic bacteria
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The possible role of anaerobic bacteria in chronic prostatitis
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Anaerobe, **4**: 133-138 (1998) IF: 1.130

XIII. Török L., **Szőke I**., Pajor L., Szöllősi J., Scultéty S.
Anaerob baktériumok szerepe férfi infertilitásban
Magyar Andrológia (accepted in 1999)

Abbreviations

CML	carboxy modified latex
OD	optical density
PAA	particle agglutination assay
BSA	bovine serum albumin
PBS	phosphat buffered saline
HAU	haemagglutinating units
EPS	expressed prostatic secretum
ETBF	enterotoxigenic *Bacteroides fragilis*
BBE	Bacteroides bile esculin
TNF	tumor necrosis factor
LPS	lipopolysaccharide
ECM	extracellular matrix proteine
SIRS	systemic inflammatory response syndrome
PY	peptone-yeast extract
BHI	brain heart infusion
CFU	colony forming unit
AA	auto-agglutination
NCTC	National Collection of Type Cultures

B. fragilis	*Bacteroides fragilis*
E. coli	*Escherichia coli*
S. haemolyticus	*Staphylococcus haemolyticus*
S. saprophyticus	*Staphylococcus saprophyticus*
S. epidermidis	*Staphylococcus epidermidis*
S. aureus	*Staphylococcus aureus*
C. trachomatis	*Chlamydia trachomatis*
T. vaginalis	*Trichomonas vaginalis*
C. difficile	*Clostridium difficile*
B. vulgatus	*Bacteroides vulgatus*

B. caccae	*Bacteroides caccae*
B. merdae	*Bacteroides merdae*
B. ovatus	*Bacteroides ovatus*
B. thetaiotaomicron	*Bacteroides thetaiotaomicron*
B. uniformis	*Bacteroides uniformis*
B. distasonis	*Bacteroides distasonis*
B. multiacidus	*Bacteroides multiacidus*
P. corporis	*Prevotella corporis*
P. prevotii	*Peptostreptococcus prevotii*
F. nucleatum	*Fusobacterium nucleatum*
M. homonis	*Mycoplasma hominis*
U. urealyticum	*Ureaplasma urealyticum*
E. faecalis	*Enterococcus faecalis*
S. α-haemolyticus	*Streptococcus α-haemolyticus*
B. urealyticus	*Bacteroides ureolyticus*

Introduction

Anaerobic pathogens have become increasingly important in clinical infection over the past decade. They generally originate from the patient's indigenous flora and are therefore associated with a variety of infections in proximity to the mucosal surfaces where they reside. These endogenous infectious processes are most frequently polymicrobial, involving aerobic and anaerobic species, where the outcome of the infection is greatly influenced by the pathogenic features of the different species involved (1). Various local and systemic factors are known to make anaerobic infections more likely:

Local tissue factors: Poor blood supply and tissue necrosis lower the local oxidation-reduction potential and favor the growth of anaerobic bacteria. Any condition that induces ischemia in an affected area of the body can predispose to anaerobic infection, including trauma, a foreign body, malignancy, surgery, diabetes, shock, colitis and vascular disease. Prior infection with aerobic or facultative organisms may likewise make the local tissue conditions more favorable for the growth of anaerobic bacteria.

Immunologic factors: Human defense mechanisms may similarly be impaired by anaerobic conditions. The ability of polymorphonuclear leukocytes to kill *Clostridium perfringens* is decreased under anaerobic conditions (2), although it is not rendered completely ineffective (3).

Among the broad spectrum of species of anaerobic bacteria in the normal flora of humans, a few exhibit marked pathogenic potential and are responsible for the majority of infections. The factors that determine the virulence of particular species are varied and probably interrelated. Just as most anaerobic infections are polymicrobial and depend on interactions of a combination of species, the virulence of a species probably depends on a combination of properties, including surface structures, metabolic functions, the ability to avoid the host's defenses, and the capacity to damage tissues. Thus, the production of each virulence factor (adhesins that attach to epithelial and red blood cells and to other bacteria, producing metabolically interdependent ecosystems; capsules that protect against phagocytosis and induce abscess formation; lipopolysaccharides (LPSs);

proteases, including those that degrade immunoglobulins and complement components; and other hydrolytic enzymes; toxin production; and resistance to antimicrobial agents) comprises only one component of virulence. Nevertheless a consideration of these factors in combination is the first step in the clarification of the mechanisms by which anaerobes cause diseases (4).

Most bacterial pathogens initiate infectious diseases by adhering to host cells. Bacterial adherence to non-phagocytic cells usually leads to extracellular colonization; however, many invasive microorganisms enter the host cells after binding to the host cell surface. After the bacterium encounters the host, the microbe must colonize a particular tissue. The microorganism may replicate at this site or move deeper into the host tissues before significant replication occurs. Damage to the host may occur at any point in this process, either because of products syntethized by the bacterium or in consequence of the harmful effects on the normal host defense mechanisms. The first step in an infectious disease (surface colonization or entry into tissues that are portals for the infection) requires specialized factors encoded by the microorganism that allow binding to host cells. Such colonization factors are not sufficient for the microorganism to cause disease, but ultimately help determine the severity of the disease and the organ system that is affected. The bacterium usually binds to the external surface of the cell and colonizes, particularly if the encounter occurs in the epithelial layers that line the oral, intestinal or urogenital tract. Such extracellular adhesion may result from direct binding of the microorganism to receptors located on the cell surface or from binding to host-encoded, secreted polysaccharides and proteins that bind to host receptors (5). Much of what is known about the adhesion of bacteria to epithelial surfaces is a result of the analysis of pili (fimbriae), which are found coating a variety of Gram-negative bacterial species (6). Recent studies demonstrate that the capsule is an important virulence factor in *Bacteroides fragilis* strains (7, 8). Pruzzo *et al.* (9) have shown the ability of piliated *B. fragilis* isolates to hemagglutinate erythrocytes and to adhere to epithelial cells. Pili-mediated adhesion is the best-studied strategy for surface colonization by pathogenic microorganisms, but it may not be the most commonly used. Gram-positive bacteria and eukaryotic microorganisms that cause infectious

diseases do not have pili. A variety of pathogenic organisms bind to extracellular host proteins that, in turn, attach to host cells (6, 10). The proteins most often bound by these microorganisms are extracellular matrix (ECM) components and include fibronectin, laminin, collagen and vitronectin. A number of pathogens colonizing various mucosal surfaces bind to sialic acid residues in mucins and to cell surface glycoproteins and glycolipids (11).The bacterial surface protein adhesins and their receptors are shown in Table I* :

I. Lectins

1. Fimbriae (pili)
   Filaments ("fuzzy layers")
   - monosaccharide inhibitors (galactose, fucose, mannose, sialic acid, etc.)
   - di- and trisaccharide inhibitors
2. Non-fimbriae
   Cell surface lectins (hemagglutinins)

II. Non-lectins

Fibronectin, collagen, etc. binding proteins

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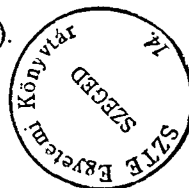
*(Torkel Wadström and Par Aleljung: Cell receptors for anaerobes and their products. In Clinical and Molecular Aspects of Anaerobes. Ed. S.P. Borriello, 1990)

Like most other organisms, bacteria require iron for cellular functions. Human and animal hosts provide an environment in which the availability of iron is limited. The majority of iron in humans is bound to iron-binding proteins such as lactoferrin and transferrin (12). Lactoferrin is present in gastrointestinal mucosal surface secretion. The clinical importance of *B. fragilis* in human anaerobic infections is well known, and the available evidence points to the existence of particular properties in the pathogenicity of these species. The adherence of *B. fragilis* and related species to ECM proteins such as fibronectin, vitronectin and laminin is another proposed factor (13, 14).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The specific form of bacterial adherence is biofilm formation. Biofilm systems produced by microbial adhesion and aggregation and mediated by the glycocalyx "slime" are of intense interest because of their significance in almost all biologic and industrial systems and their profound effect on humans in health and disease, especially in resistant or chronic biomaterial/damaged tissues centered infections. Shortly after attachment and adhesion, as bacterial growth and propagation accelerate and if colony conditions are optimal, extracellular polysaccharide polymers are produced. These polymers enhance adhesion to surfaces and function in the coaggregation of daughter cells or cells of other microbial species in consortia formation and polymicrobial infection. The biofilm matrix formed by the exopolysaccharide polymers not only serves as an adhesive mechanism, but also appears to be related to virulence.

The pathogenesis of damaged tissue-centered infection can best be understood as a unique process by cataloguing its common features: (i) the colonization of substrata by adhesive biofilm-forming bacteria, (ii) the presence of biomaterial or damaged tissue, (iii) the initiation of infection by small bacterial inocula, (iv) bacterial biofilm-mediated resistance to host defense mechanisms and antibiotic therapy, (v) opportunistic pathogens become virulent organisms because of the presence of a biomaterial substratum, (vi) infections are frequently polymicrobial (15).

The bacterial biofilm theory may help explain some of the problems linked to our understanding of the nature of urinary tract infections. Biofilm formation may play a role in catheter-associated infection and prostatitis (16). Infectious forms of prostatitis occur after puberty and progressively increase in incidence thereafter (17). In acute and chronic bacterial prostatitis, urinary tract pathogens (members of *Enterobacteriaceae* and *Pseudomonas* spp.) are causally involved. More than one species can often be simultaneously demonstrated in the prostatic secretion. The role of anaerobic bacteria in chronic bacterial prostatitis has not been investigated widely, but a few communications have reported attempts to culture prostatic secretum in an anaerobic environment (18,19). There are a number of reasons why prostatic infections tend to be refractory to the traditional courses of therapy. The first is the variable penetration of the active drug into the prostatic parenchyma for most antibiotics (20,21). The second is that the infection may be

sequestered in a glycoprotein matrix secreted by the bacterium, which may inhibit the action of antibiotics by limiting their access to the bacteria (22). Lastly, prostatic stones may become a nidus for infection by harboring bacteria within the interstices of the stone, thereby affording protection of bacteria against the antibiotic that is being utilized. All of these factors complicate the treatment process for chronic bacterial prostatitis, making it difficult to decide on the appropriate time course of therapy.

The oral cavity harbors hundreds of different microbial species. This complex microflora displays a great quantitative and qualitative variation at different locations within the oral cavity. The number and the composition of the anaerobic bacteria present as the normal flora of the mouth may change significantly in different intraoral disorders (23). Both local and systemic infections may complicate the morbidity of patients with oral malignant neoplasms, particularly those presenting intraorally.

An other important pathogenic factor of different bacterial species is the toxin production. *B. fragilis* is the anaerobic species most frequently isolated from infections in humans, but it was not implicated as a cause of intestinal diseases until the mid-1980s. The description of an enterotoxin produced by *B. fragilis* suggested a possible new role of this microorganism. In 1984 Myers *et al.* isolated enterotoxigenic strains of *B. fragilis* (ETBF) from the faeces of neonatal lambs with diarrhoea (24). Studies in humans revealed that individuals with or without diarrhoea can harbor ETBF srains in the intestine, but few data are available as concerns the distribution of ETBF strains among strains originating from infectious processes (25, 26). *B. fragilis* is primarily an extraintestinal pathogen, and the cytotoxic potential of the toxin produced by some of the isolates may suggest that as a pathogenic factor it could participate in the invasive and destructive processes of infections caused by these bacteria (27, 28).

In the past few years it has become accepted that much of the systemic endotoxin involved in the pathogenesis of systemic inflammatory response syndrome (SIRS) or sepsis in the severely ill patient originates in the bowel. Bacteria, their constituents and products may translocate during episodes of shock-induced gut ischemia to the portal circulation, where lipopolysaccharides (LPS) and possibly other biologically active molecules interact with Kupffer's cells, the macrophages

of the liver, and the mononuclear cells in the blood stream. Many of the subsequent pathophysiological changes characteristic of SIRS result from the induction of the most important proinflammatory cytokines, such as TNF-α and IL 1β. These "proximal" cytokines and also endotoxin stimulate the production of the late or distal cytokines, such as IL-6 and IL-8 (29, 30). In a variety of models, both *in vivo* and *in vitro*, it has been reported that the endotoxicity of *Bacteroides* LPSs is 10000 to 10-fold lower than the activity of LPSs of the members of the family *Enterobacteriaceae* (31). This finding, together with the known differences in number of *Bacteroides* and *Escherichia coli* or other facultative Gram-negative bacteria present in the gut, might suggest (even alloving for much lower biological activity) that within the gastrointestinal tract the pool of potential endotoxin may originate as much from the *Bacteroides* strains as from *E.coli.*

Aims of this studies were

1. To examine the binding of different ECM proteins to a number of *Bacteroides* and other anaerobic bacterial strains originating from various infectious processes or from the stools of healthy individuals, by using different methods.
2. To elucidate the role of anaerobic bacteria as causative organisms in chronic bacterial prostatitis. We have investigated their incidence in parallel in the urethral sample and prostatic secretum obtained after prostatic massage of chronic prostatitis patients where infecting organisms could previously not be identified.
3. To characterize the biofilm flora of patients with oral carcinoma before any treatment.
4. To test *B. fragilis* isolates originating from faecal samples and from infectious processes for their ability to cause cytotoxic changes in HT-29 cells, and to detect the presence of free toxins in the faeces from patients with diarrhoea.
5. To establish whether or not intact *Bacteroides* strains belonging in different species, isolated from severe human infections or from the faeces of healthy persons, are capable of stimulating the release of TNF and IL-6 by separated human mononuclear cells and by whole blood culture.

Materials and Methods

1. Examination of the binding of anaerobic bacteria to extracellular matrix proteins

1.1. Bacterial strains

Examinations were performed on 40 *B. fragilis* and 66 other *Bacteroides* isolates from materials obtained after abdominal surgery, from peritoneal fluid, from deep-seated wounds and from pus from infections after gynaecological surgery, on 101 *Bacteroides* isolates from faeces of healthy subjects and on 10 different anaerobic isolates (*Fusobacterium nucleatum* two strains, *Peptostreptococcus prevotii* three strains and *Prevotella corporis* two strains) from tonsils removed at tonsillectomy; and three *Clostridium difficile* strains. *Staphylococcus aureus* Cowan I, *S. haemolyticus* E 2498 and *S. haemolyticus* SM 131 were used as positive controls, and *S. saprophyticus* TW 111 and *S. epidermidis* 3380 as negative controls for latex agglutination assays. *Helicobacter pylori* strain NCTC 11637 was the positive control for the particle agglutination assay (PAA) with fetuin- and asialofetuin-coated beads.

1.2. Culture media

The growth conditions may affect the binding of ECM proteins such as fibronectin and collagen-I to bacterial cells (32). Accordingly, for variation of the growth conditions, anaerobic strains were cultured on peptone-yeast extract (PY) or on brain-heart infusion (BHI) agar supplemented with 5% horse blood. BHI broth and Wilkins-Chalgren broth were used in parallel. The bacteria were incubated for 48 h in an anaerobic jar at 37 °C or at 30 °C.

1.3. Preparation of latex reagents

Latex particles coated with different proteins were prepared according to Naidu *et al.* (32). Briefly, 1 ml of a latex particle suspension (0.8 µm diameter) was mixed with 3 ml of 0.17 M glycine - NaOH buffer (pH 8.2) and centrifuged at 4500 g for

5 min, and the pellets were resuspended in 3 ml of the same buffer. Highly purified protein (100 μg) was added, and the mixtures were kept at 30 °C for 12 h on a horizontal shaker at 50 rpm. The mixtures were centrifuged at 9 200 g for 5 min at 20 °C, and the supernatants were discarded. The pellet was resuspended in 2 ml of the glycine buffer containing merthiolate (0.01%) and bovine serum albumin (BSA) (0.05%) and kept at 4 °C for 12 h.

The fetuin- and asialofetuin-coated beads were prepared by using 100 μl of CML (0.8 μm) particles, which were washed twice with 0.1 M phosphate buffer (pH 8.1) and suspended and incubated overnight at 4 °C in 2 ml of phosphate buffer containing 4 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The suspension was centrifuged (1200 g, 10 min, 4 °C), and the particles were suspended in phosphate buffer containing fetuin or asialofetuin (1mg/ml) and icubated overnight at 4 °C. The suspensions were centrifuged (12 000 g, 10 min, 4 °C), and the protein-coated CML particles were washed four times with phosphate buffer and suspended in the same buffer to a final concentration of 1% total beads, and stored at 4 °C until used.

1.4. Particle agglutination assay

The 217 isolates were grown on various media for 48 h. Bacterial colonies were suspended and washed once in 0.02 M potassium phosphate buffer (pH 6.8). Bacterial cells were resuspended in the same buffer to approximately OD 1 at 540 nm and immediately subjected to the PAA (32). The strains were tested for autoaggregation by mixing one drop of phosphate buffer and one drop of bacterial cell suspension. Latex beads coated with BSA were also tested as a negative control.

1.5. Heat treatment and proteolytic digestion

For enzyme treatment, bacteria were washed twice in PBS, and the cell density was adjusted to OD 1. Proteinase K and pronase E were dissolved (1 mg/ml) in phosphate-buffered saline (PBS) (pH 7.2), and trypsin in PBSA (pH 7.2). Bacterial suspensions and these enzyme solutions were mixed (1:1). For heat treatment, bacteria were washed twice in PBS, the concentration of bacteria was

adjusted to OD 1, and they were incubated in a water bath at 40 °C, 60 °C, 80 °C or 100 °C for 20 min.

1.6. Particle agglutination inhibition assay

100 μl of inhibitor (fibronectin, 0.1 M D-galactose, D-glucose, L-fucose, N-acetylgalactoseamine or mucin) was preincubated with an equal volume of bacterial cell suspension for 30 min at 20 °C and then mixed with the PAA reagents. Potassium phosphate buffer (pH 6.8) was used as a negative control.

1.7. Hemagglutination and hemagglutination inhibition assay

Harvested bacterial cells were suspended in PBS (pH 7.2) to a final colony count of 10^9 cells/ml. Equal volumes (50 μl) of a bacterial suspension in PBS and a 1% erythrocyte suspension were mixed in the wells of a microtitration plate and allowed to settle for 1-2 h at 20 °C. PBS was used as a negative control. For the hemagglutination inhibition assay, the bacterial cell suspensions were diluted with PBS to give 4 HAU (hemagglutinating units). The solutions of inhibitors were also prepared in PBS (pH 7.2) at concentrations of 1 or 2.5 mg/ml. Inhibition tests were performed in the wells of a microtitration plate with erythrocyte (1%) suspensions. Bacterial cell suspensions (50 μl) were mixed with 30 μl of inhibitor solution for 1 h; 50 ul of erythrocyte suspension was then added, and the suspension was alloved to settle at room temperature for 1-2 h. Controls contained 30 μl of PBS instead of inhibitor solution.

1.8. ^{125}I-labelled protein binding assay

Fibronectin, collagen-I and lactoferrin were labeled with iodine according to a modified chloramine-T method, using Iodobeads (33). The labeled proteins were kept at 4 °C and used within 3 weeks. Radiolabeled proteins were diluted to approx. 30 000 cpm/50 μl in PBS (pH 7.2) containing 1% BSA and added to 100 μl of a bacterial cell suspension. After thorough mixing, samples were incubated at room temperature for 1 h. The binding reaction was stopped by the addition of 2 ml of ice-cold PBS containing 0.1% Tween 20. Supernatants were aspirated after centrifugation at 3 000 rpm for 15 min and the radioactivity of the pellets

was measured in a gamma-counter. For each experiment, negative control test tubes with all ingredients except bacteria were included and the radioactivity was determined. *S. aureus* Cowan I was used as positive control.

1.9. Electron microscopy

Selected *B. fragilis* strains were incubated for 48 h in Wilkins-Chalgren anaerobic broth in an anaerobic jar, and on PY agar supplemented with horse blood (5%). The bacteria were washed carefully in 0.02 M phosphate buffer (pH 6.8) and centrifuged at 1600 g for 15 min. For examination of the bacterial cell surface structures by electron microscopy, negative staining with phosphotungstic acid (2%, pH 7.2) for 1 min was used.

1.10. Chemicals

Vitronectin and fibronectin were purified from human plasma according to Vuento *et al.* (33). A collagen preparation (Vitrogen 100R, containing 95% type-I and 5% type-III collagen, lot no. 87H183) was purchased from Collagen Corporation, Paolo Alto, CA. Collagen type I (Sigma) was also tested. Pronase E, proteinase K, trypsin, ovalbumin, fetuin, asialofetuin, D-mannose, L-fucose and mucin (Sigma M1778, lot 112H7025) were purchased from Sigma Chemicals, St. Louis, MO, USA. BSA was obtained from Boehringer Mannheim GmbH. Iodobeads were from Pierce Chemical Company (USA), and latex beads (0.8 µm diameter) from Difco Laboratories. Carboxy-modified latex (CML) beads (0.499 µm diameter) were obtained from Seradyn, Indianapolis, USA. Merthiolate was from Kebo Lab. AB, Stockholm, Sweden.

2. Examination of the role of anaerobic bacteria in chronic prostatitis

169 male patients (aged 25-56 years), with clinical symptoms of chronic prostatitis resistant to empiric quinolone therapy, were involved in this study. The methods applied to establish the clinical diagnosis of chronic prostatitis included ultrasonographic and microbiological examinations. None of the patients had received any treatment for at least 2 weeks before the collection of the samples.

Urethral specimens and the expressed prostatic secretum (EPS) were collected with cotton tipped swabs. The swabs were transported to the laboratory in an anaerobic transport medium (Stuart medium). A direct smear of the EPS was also examined after Gram staining, and leukocytes were counted per high-power field. Specimens were cultured on Columbia agar (Pasteur Sanofi) supplemented with 5% cow blood for 48 h at 37 °C in 5% CO_2 for aerobic and facultative anaerobic bacteria, and on Columbia agar supplemented with 5% cow blood, 100 µg/ml vitamin K_1 and 300 µg/ml cysteine for 6 days at 37 °C in an anaerobic chamber (Bactron, Sheldon Man. Inc., Mansfield, USA). For the detection *Gardnerella vaginalis,* the same agar supplemented with 5% human blood was used, with anaerobic incubation. The presence of *Chlamydia trachomatis, Mycoplasma/Ureaplasma*, *Trichomonas vaginalis* and *Candida* species was recorded. As controls, urethral samples from 40 male patients with clinical symptoms of urethritis, and seminal fluid from 30 healthy subjects, were cultured in a similar way. The isolated aerobic and anaerobic bacteria were identified by conventional methods.

3. Examination of the bacterial flora on oral tumor surfaces

Samples were obtained with a cotton swab from 1 cm^2 of lesion surface centers and contiguous healthy mucosa from 21 patients (20 male and 1 female patient, mean age 52.8 years) with oral cancer before chemotherapy or any other tumor treatment. All lesions were keratinizing squamous cell carcinomas.

Samples were transported to the microbiological laboatory in 1 ml prereduced BHI broth immediately after collection and cultured within 1 h. After careful shaking (vortex), the suspensions were diluted (10^{-1}-10^{-6}) and 100 µl of each dilution and 100 µl of undiluted specimen were plated immediately on the surface of selective and non-selective media:

- for the culturing of aerobic bacteria

non-selective media: Columbia agar supplemented with 5% cow blood
Chocolate agar

selective for streptococci: Mitis Salivarius Agar (Difco Laboratories)

fungi: Sabouraud Dextrose Agar (Difco Laboratories)

Gram-negative bacteria: Endo agar

- for the culturing of anaerobic bacteria

non-selective medium: Columbia agar supplemented with 5% cow blood, hemin, vitamin K_1

selective for *Veillonella* spp.: Veillonella agar (Difco Laboratories)

Actinomyces spp.: CFAT agar supplemented according to the given procedure

Lactobacillus spp.. Rogosa agar (Oxoid)

The samples were cultured for aerobic bacteria at 37 °C in a 5% CO_2-containing environment for 48 h, and for anaerobic bacteria in an anaerobic chamber (Bactron, Sheldon Man. Inc., USA) for 6 days. The selective agar plates for Gram-negative aerobic bacteria were incubated at 37 °C for 48 h. The Sabouraud agar was incubated at 37 °C for 24 h and at room temperature for another 5 days. After incubation, colonies were counted. The isolated bacteria were identified by means of the ATB identification system (bioMerieux, Lyon, France) or by GLC.

4. Toxin production of *Bacteroides fragilis* strains

Onehundred and thirty-four *B. fragilis* strains were collected during a period of 6 months in 1995-96. Fourty were isolated from the faeces of outpatients with diarrhoea where no other causative agents were found, 20 from the faeces of healthy subjects and 74 from different clinical samples, including soft-tissue infections and intra-abdominal abscesses after surgery. The toxin-positive control strain NCTC 11295, a metronidazole-resistant clinical isolate, and AN 209, a faecal isolate originating from Annalisa Pantosti (Rome, Italy), were also tested. To assess enterotoxin production, each *B. fragilis* strain was grown overnight at 37 °C in BHI broth supplemented with 0.5% yeast extract and 0.005% hemin in an anaerobic chamber (Bactron, Sheldon Man. Inc., USA). The cultures were centrifuged at 8000 g and the supernatants were frozen immediately and kept at -20 °C until used. HT-29 cells were grown according to Pantosti *et al.* (35). For the cytotoxicity assay, HT-29 cells were distributed into a 24-well cell culturing plate and allowed to grow for 2-3 days until discrete clusters of cells were visible.

Before the assay, the cell culture medium was removed and fresh medium without serum was added to each well. Two-fold dilutions of the filtered bacterial culture supernatants were then inoculated into the wells. The plates were incubated at 37 ^{o}C in a 5% CO_2-containing environment, and examined after 2, 4 and 18 h for the presence of the typical toxin-induced cytopathic changes. The faecal samples of 50 patients with diarrhoea were selected according to their negative culture results for common enteric pathogens such as *Salmonella*, *Shigella*, *Yersinia*, *Campylobacter*, *Aeromonas* and *C. difficile* and negative latex agglutination tests for rota virus and adenovirus. To detect the free toxin of *B. fragilis*, the stool samples were diluted 1:1 in phosphate buffer and centrifuged at 4 000 rpm for 10 min. 100 μl of the filtered supernatant was added to the HT-29 cell-containing wells and tested in the same way as culture supernatants of the isolated *B. fragilis* strains. To investigate the presence of ETBF in the stool specimens of patients with diarrhoea, the toxin-positive faeces were plated on Bacteroides Bile Esculin (BBE) agar and the plates were incubated anaerobically for 3 days. *B. fragilis* isolates were identified through the use of Rapid ID 32A (bioMerieux, Lyon, France) and were further tested for the production of enterotoxin by use of the HT-29 cell line.

5. Examination of TNF induction

5.1. Bacterial strains and culture methods

Thirty-four *Bacteroides* strains were isolated from severe infections after abdominal surgery. Ten *B. fragilis*, 6 *Bacteroides ovatus*, 8 *Bacteroides vulgatus* and 10 *Bacteroides thetaiotaomicron* strains were involved in the study. Four strains (one *B. fragilis*, one *B. vulgatus*, one *B. caccae* and one *B. merdae*) were obtained from faecal samples of healthy persons. As control, *Staphylococcus aureus* (Wood) was used. To stimulate human mononuclear cells (MN), colonies were freshly inoculated into BHI (Pasteur-Sanofi) broth and incubated in an anaerobic environment (anaerobic chamber: Bactron, Sheldon Man. Inc., Mansfield, USA) for 24 h at 37 °C. All *Bacteroides* strains were cultured in parallel in BHI broth supplemented with 5% horse serum. The control *S. aureus*

strain was cultured in Muller-Hinton broth (Pasteur-Sanofi) for 24 h at 37 °C. For the TNF and IL-6 induction experiments, heat-inactivated (100 °C for 20 min) washed cells were used (10^9 CFU/ml).

5.2. Isolation and stimulation of human mononuclear cells

Mononuclear cells were prepared by centrifugation of heparinized venous blood from healthy donors by density centrifugation on a Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient (36). The cells at the interface were removed and washed three times by centrifugation at 400 g for 10 min, and finally resuspended at the appropriate concentration in RPMI-1640 (Gibco, Grand Island), containing 10% foetal calf serum (Gibco) and supplemented with 100 ug/ml gentamycin and 100 ug/ml penicillin (37) to achieve 5 x 10^6 cell/ml. Heparinized peripheral blood without any separation was also used and is referred to as whole blood. 0.1 ml aliquots of the heat-inactivated *Bacteroides* cell suspensions were added to 1 ml mononuclear cells and also to 1 ml whole blood cultures. As controls of stimulation of TNF or IL-6 production heat-inactivated cells of *S. aureus* (Wood) and *E. coli* O128 B12 LPS (10 μg/ml) (Sigma) were also used. After incubation for 18 h at 37 °C (5% CO_2), the cells were centrifuged for 15 min at 700 g. TNF was titrated in a bioassay on the cell line WEHI 164 clone 13 (38). IL-6 was measured via its proliferative action on the IL-6-dependent mouse hybridoma cell line B-9 (39). The activities were calibrated against rh TNF (Genzyme Corp. Cambridge) or rh IL-6 (Sigma Aldrich, Munich), respectively.

5.3. Isolation and characterization of LPS of *B. fragilis* strain No. 7

The strain was cultured in 5 x 1000 ml BHI broth with or without supplementation with 5% horse serum in an anaerobic environment for 48 h. The extraction of LPS from the acetone-dried cells was carried out by the aqueous phenol method of Westphal and Luderitz (40). The raw extract was purified by dialysis and ultracentrifugation (3x4 h, 100 000 g). The sediment (LPS) and the supernatant of the first ultracentrifugation (L1 fraction) were freeze-dried. The native LPSs were freed from protein contamination by treatment with proteinase K (20 ug/ml) at 65 °C for 2 h. Proteinase K was removed by two washes with

pyrogen-free water at 100 000 g. The lyophilized LPSs and L1 fractions and also the control *E. coli* O128 B12 LPS were further used for the induction of TNF and IL-6 in a concentration of 10 ug/ml. The LPSs of *B. fragilis* strain (No 7) and the control *E.coli* 536 were analyzed by PAGE. The samples were dissolved to a concentration of LPS 1 mg/ml in the SDS-2-mercaptoethanol solubilisation buffer of Laemmli (41) and heated at 100 °C for 3 min. 10 ul samples were applied to the gel and stained with silver by the method of Tsai and Frasch (42).

Results and Discussion

1. Results of the binding of anaerobic strains to ECM (papers I and IV)

Altogether 207 *Bacteroides* strains and 10 other anaerobic bacteria were grown on different media at different temperatures and were tested in PAA by using latex particles coated with fibronectin or collagen-I. PY blood agar proved to be the optimum culture medium for *Bacteroides*, because only 34% of the strains displayed autoaggregation and the binding of the strains was stronger than on culturing in other media (BHI broth and agar, Wilkins-Chalgren broth) (Table II). There were no differences in the binding of the strains if they were incubated at 30 °C or at 37 °C.

Table II Influence of culture medium on fibronectin and collagen binding of different anaerobic strains examined by PAA

	Strains giving positive PAA (%) with		Strains exhibiting
Culture medium	Fibronectin	Collagen-I	AA(%)
PY blood agar	46	55	34
BHI blood agar	32	49	46
BHI broth	29	44	42
Wilkins-Chalgren broth	30	44	43

AA, auto-agglutination; BHI, brain-heart infusion; PY, peptone-yeast extract; PAA, particle agglutination assay

Twohundred and seven *Bacteroides* strains and 10 other anaerobic bacterial strains were examined with different proteins immobilized on latex beads (Tables III and IV). The binding of fibronectin and collagen-I was stronger to *B. fragilis* strains isolated from infections and from faeces of healthy individuals than to strains of other *Bacteroides* species. No significant differences were observed in the binding of the different proteins to the other *Bacteroides* strains isolated from infections or from healthy subjects. The binding of vitronectin was less common, but was always observed to accompany fibronectin binding. Some of the anaerobic strains displayed agglutination with ovalbumin-coated beads, which is why beads coated with BSA were used as negative control. Of the 10 other anaerobic strains, only one *Clostridium difficile* strain gave a strong positive PAA reaction with fibronectin, collagen-I or vitronectin. Collagen-I bound to a *Prevotella corporis* isolate (Table V).

Table III Binding of 207 *Bacteroides* spp. to matrix proteins in the PAA test

Species		Number of strains showing				AA
		fibronectin binding		vitronectin binding		
		+	-	+	-	
Isolated from infections						
B. fragilis	(40)	28	0	18	10	12
B. thetaiotaomicron	(15)	3	12	1	14	0
B. ovatus	(15)	6	8	3	11	1
B. vulgatus	(15)	14	0	6	8	1
B. uniformis	(10)	0	10	0	10	0
B. distasonis	(9)	2	7	0	9	0
B. multiacidus	(2)	0	2	0	2	0
Isolated from faeces of healthy subjects						
B. fragilis	(25)	15	2	8	9	8
B. thetaiotaomicron	(9)	3	6	0	9	0
B. ovatus	(12)	0	12	4	8	0
B. vulgatus	(18)	6	2	1	7	10
B. caccae	(15)	1	14	2	13	0
B. uniformis	(3)	0	3	0	3	0
B. merdae	(3)	0	3	0	3	0
B. stercoris	(16)	0	15	0	15	1

AA, auto-agglutination; PAA, particle agglutination assay

Table IV Binding of 55 *Bacteroides* strains to collagen-I by PAA

Strains		Number of strains giving positive PAA reactions with collagen-I		AA
		+	-	
From infections				
B. fragilis	24	15	0	9
B. ovatus	6	2	0	4
B. thetaiotaomicron	2	1	0	1
B. multiacidus	1	1	0	0
B. vulgatus	3	1	0	2
From faecal samples				
B. fragilis	13	9	2	2
B. ovatus	1	1	0	0
B. vulgatus	2	2	0	0
B. merdae	2	2	0	0

PAA, particle agglutination assay; AA, auto-agglutination

Table V Binding of 10 other anaerobic strains to ECM by PAA

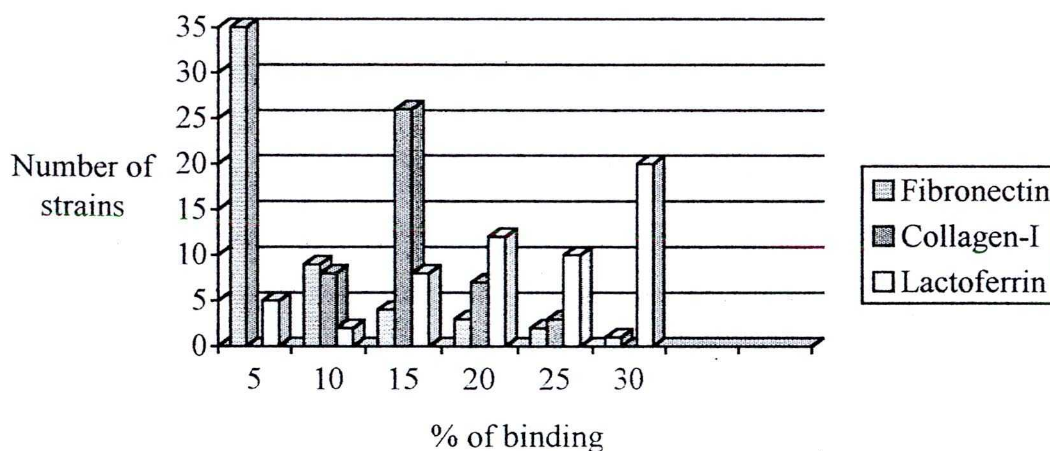
Strains	Number of strains giving positive PAA reactions with fibronectin		collagen-I		vitronectin		AA
	+	-	+	-	+	-	
C. difficile (3)	1	1	1	1	2	0	1
P. corporis (2)	0	1	1	0	0	1	1
P. prevotii (3)	0	3	3	0	0	3	0
F. nucleatum (2)	0	2	0	2	0	2	0

ECM, extracellular matrix protein; PAA, particle agglutination assay; AA, auto-agglutination

None of the examined strains bound to fetuin- or asialofetuin-coated beads. The binding of fibronectin in the PAA was completely inhibited by preincubation of the bacterial suspension with soluble fibronectin (1 mg/ml). Other glycoproteins or carbohydrates, such as sucralfate, suramin, fragmin, protamine, polylysine, dextran sulphate 500, pentosan sulphate and heparin, did not inhibit the binding of fibronectin to the cells. The cell surface structures of the *Bacteroides* strains were sensitive to all proteases used (pronase E, proteinase K and trypsin).

The binding of ^{125}I-labeled fibronectin, ^{125}I-labeled collagen-I and ^{125}I-labeled lactoferrin to 55 *Bacteroides* strains was also tested. Only 29% of the strains exhibited strong binding (15-30%) to ^{125}I-fibronectin. The binding of ^{125}I-collagen-I to the *Bacteroides* strains was similar to that observed in the PAA. 70% of the strains exhibited a binding of 10-15% to ^{125}I-labeled collagen-I, in parallel with strong agglutination in the PAA. The binding of ^{125}I-lactoferrin was particularly strong (30-35%) for 20 *B. fragilis* isolates (Fig. 1).

Fig. 1. Binding of 55 *Bacteroides* strains to ECM by radiolabeling method



An important early event in the development of a variety of bacterial infections is the adherence of the pathogens to epithelial and endothelial cell surfaces. Connective tissue proteins such as collagen, laminin and serum-derived fibronectin and vitronectin may all be involved in a tissue colonization process in surgical and other infections (33). The binding of fibronectin and collagen to different aerobic bacterial pathogens has been tested fairly frequently (10,11). Far

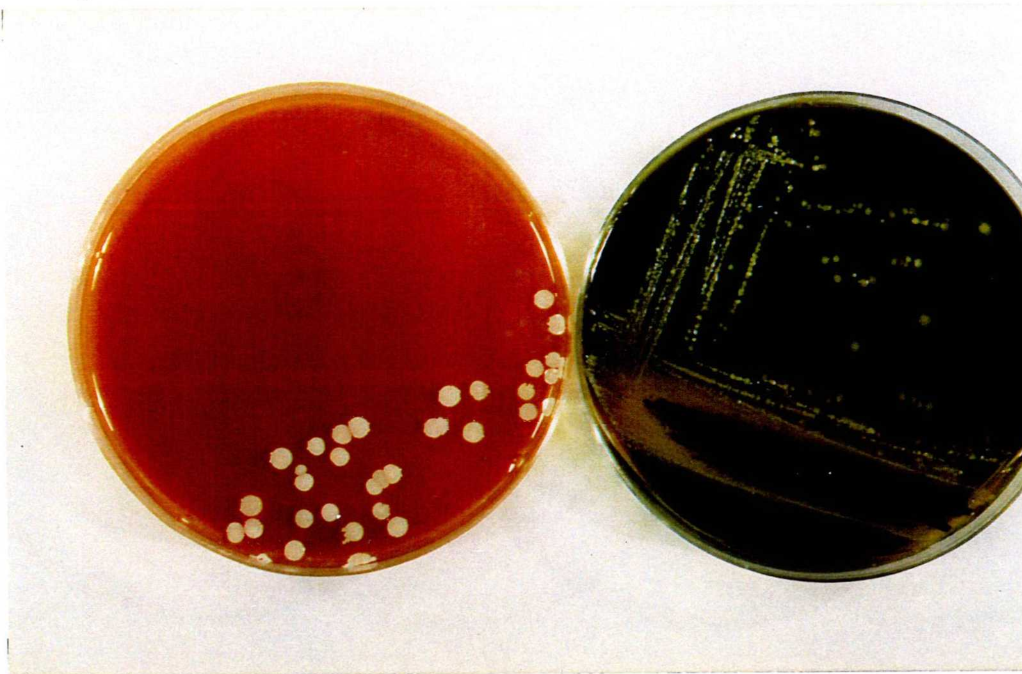
fewer data are available on the binding of vitronectin (10,13,14). The binding of different matrix proteins to *S. aureus* and various coagulase-negative staphylococci has been investigated most often, demonstrating the important role of these interactions in special infectious processes (11,44). The binding of ECM proteins to different bacterial cells may have a dual role in host-parasite interactions. The different cell surface-binding components specific for the various ECM proteins may be involved in the colonization of wounds and other tissue lesions, but they may also aid indigenous flora such as lactobacilli in the vagina and *B. vulgatus* in the colon to establish themselves on mucosal surfaces (13,45). In the present study, the strains of different species of *Bacteroides* exhibited different capacities to bind ECM proteins. Similarly as already demonstrated for other bacterial species, different culture media and growth conditions influence the expression of cell surface proteins of *Bacteroides* species. As concerns the growth of the strains on the surface of PY agar plates supplemented with horse blood proved to provide the best conditions for the expression of surface-binding sites for *Bacteroides* strains. *B. fragilis* is considered to be the most frequent pathogen involved in polymicrobial infections after surgery. The binding of different matrix proteins to *B. fragilis* strains, as demonstrated by means of PAA and the ^{125}I-labelled protein binding assay, suggests that for these strains the specific binding may be implicated in their adherence to epithelial surfaces, similarly to the processes observed with other pathogens (10, 11, 46-49). The results of this study indicate that *Bacteroides* strains contain components that recognize fibronectin and collagen type I (both immobilized and soluble forms), which can each bind effectively to members of the *Bacteroides* genus. Protease treatment of bacterial cells resulted in complete loss of the reactivity to fibronectin in the PAA, indicating the proteinaceous nature of the respective cell surface receptors. All strains which were isolated from infectious processes and did not exhibit autoaggregation proved to adhere to fibronectin and collagen-I in the PAA. Some of the *B. fragilis* strains isolated from faeces also had binding sites for fibronectin and collagen-I. On the other hand, *B. vulgatus* strains, much less frequently involved in infectious processes, were also found to possess a binding site for fibronectin. This means that the

adherence of *Bacteroides* species to fibronectin and to other ECM proteins is not necessarily connected with their pathogenicity, but may be the critical step for tissue invasion, especially if the adherence event induces other reactions possibly leading to the degradation of basement membranes. This study revealed a high level of binding of ^{125}I-lactoferrin to *Bacteroides* strains isolated from infectious processes or from the faeces of healthy individuals. Further studies are needed to determine whether the lactoferrin binding of *Bacteroides* strains may be of importance in the infectious processes they cause.

2. Microbiological results on patients with chronic bacterial prostatitis (papers III, V, VIII and XIII)

The prevalence of clinical symptoms of prostatitis is known to be considerably higher than previously reported, afflicting between 25 and 50% of all adult men. During the examination of chronic bacterial prostatitis usually, little attention is paid to anaerobic bacteria as they are sensitive to transportation and culturing, and differentiation is difficult, costly and time- consuming.
The culture results of these studies demonstrated that 28% of the patients with prostatitis exhibited high colony counts of Gram-positive and Gram-negative anaerobic bacteria ($>10^7$ CFU/ml) (Fig. 2). This mixed anaerobic flora was similar to the flora found in women with bacterial vaginosis (Fig. 3). Of the 169 examined patients, 8 (5%) were positive for both aerobic and anaerobic bacteria. The most frequently isolated anaerobic, Gram-positive bacteria were *Peptostreptococcus* spp. and *Propionibacterium* spp., and the most frequent Gram-negative bacteria were *B. ureolyticus*, *Prevotella* spp. and *Porphyromonas* spp. Altogether, 299 anaerobic bacteria were isolated from 56 patients, which means an average of 5.34 anaerobic bacteria/positive patient. *C. trachomatis* in 1%, *M. hominis* in 5%, *U. urealyticum* in 3% and *Candida* spp. in 3% were isolated. In 36% of the patients, only aerobic bacteria were cultured. The most frequently isolated aerobic bacteria were *E. coli* and other *Enterobacteriacae*, *E. faecalis*, *Proteus* spp. and *Providencia* spp. The remaining 19% were completely negative for both aerobic and anaerobic bacteria (Fig. 4.).

Fig. 2. Aerobic and anaerobic cultures of EPS from a patient with chronic prostatitis



aerobic culture anaerobic culture

Fig. 3 Mixed anaerobic bacteriumflora of EPS from patients with chronic prostatitis

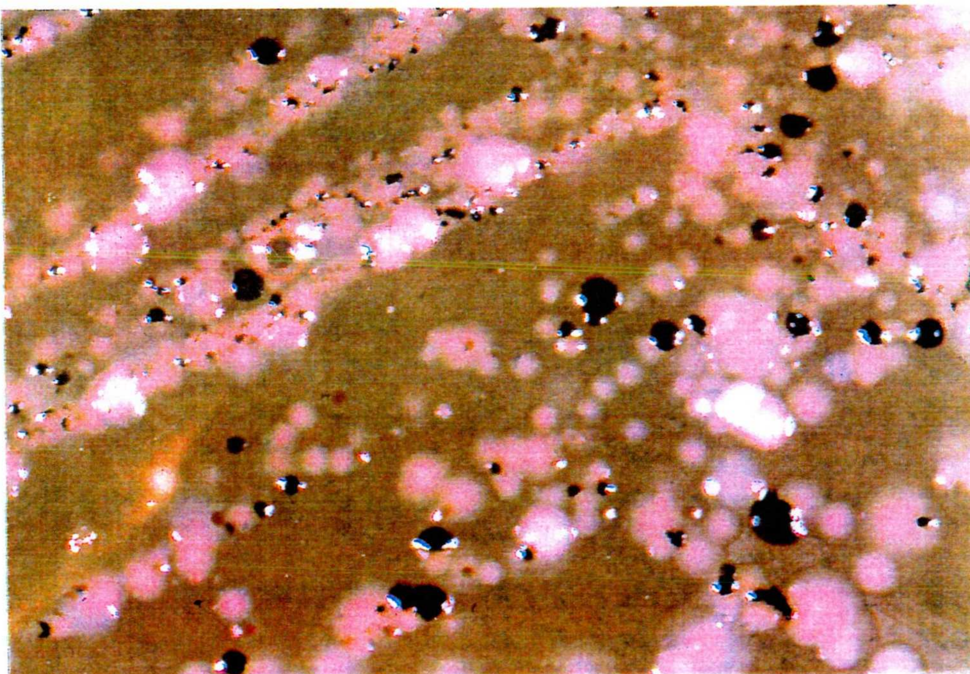
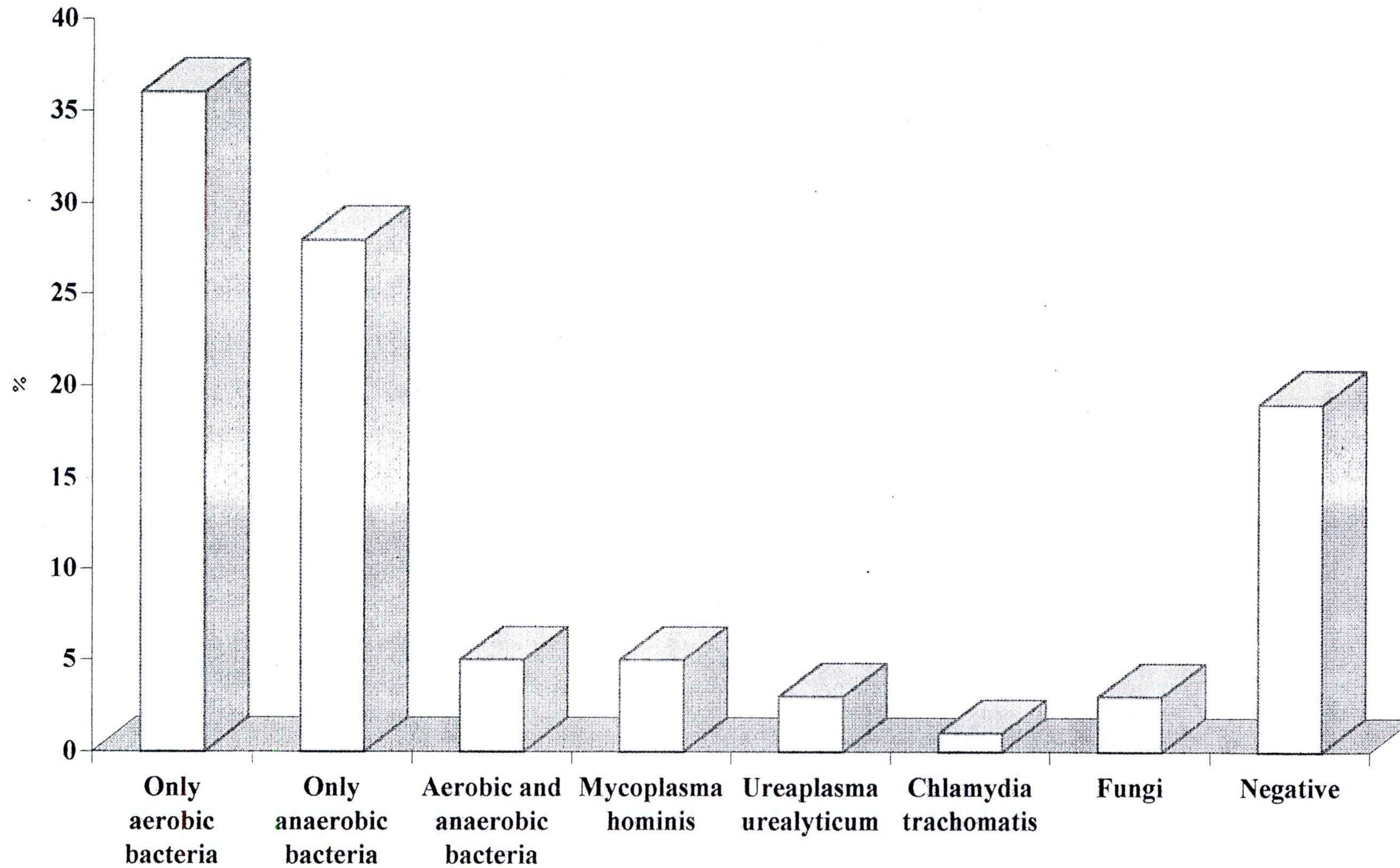


Fig. 4 Culture results on EPS/urethral samples from patients with chronic prostatitis



The distribution of aerobic and anaerobic bacteria isolated from the urethral sample and/or EPS from the patients yielding positive culture results is to be seen in Tables VI-VII.

Table VI Distribution of aerobic species isolated from urethral discharge and/or EPS from chronic prostatitis patients

Species	No. of isolates
Gram-positive aerobes	
Staphylococcus epidermidis	18
Streptococcus α-haemolyticus	14
Corynebacterium spp.	7
Enterococcus faecalis	16
Gram-negative aerobes	
Citrobacter freundii	1
E. coli	28
Proteus spp.	9
Klebsiella spp.	19
Providencia spp.	10
Total aerobes	122

Table VII Distribution of isolated anaerobic species from the samples of examined patients

Gram-positive anaerobes	
Peptostreptococcus spp.	51
Propionibacterium spp.	31
Bifidobacterium spp.	11
Eubacterium spp.	9
Actinomyces spp.	8
Clostridium spp.	5
Gram-negative anaerobes	
Bacteroides ureolyticus	28
Prevotella spp.	36
Porphyromonas spp.	35
Veillonella spp.	23
Bacteroides spp.	32
Fusobacterium spp.	28
Tissierella spp.	1
Mobiluncus spp.	1
Total anaerobes	299

All patients who gave positive EPS culture results for anaerobic bacteria or for mixed aerobic/anaerobic bacteria underwent 3 to 6 weeks of antibiotic therapy against the anaerobic bacteria found in the specimens. Amoxicillin/clavulanic acid was applied in a dose of 3x375 mg/day or clindamycin in a dose of 3x150 mg/day. During the treatment the patients were followed up: they were seen again after 3 weeks and 6 weeks. Following the completion of therapy, ultrasonographic examinations and culture procedures after prostatic massage were repeated. The cultures were then negative for anaerobic bacteria or only Gram-positive anaerobic bacteria of normal flora (*Peptostreptococcus* spp. and *Propionibacterium* spp.) were growing in low number ($<10^2$ CFU/ml). In parallel with the negative culture results, a decrease (32 patients) or total elimination (24

patients) of the symptoms was detected, but in some cases the transrectal ultrasonographic examination revealed residual abnormalities in the prostatic glands for weeks after the treatment. The patients who were negative for all pathogens examined were treated according to the treatment protocol of prostatodynia.

As concerns the two control groups, the urethral samples from 40 men with typical symptoms of urethritis were cultured similarly. 25 of these patients were infected by aerobic bacteria, either alone (18 cases) or together with anaerobes (7 cases). Only 2 patients displayed anaerobic bacteria alone. Three patients exhibited *U. urealyticum,* and 3 *C. trachomatis* (Table VIII).

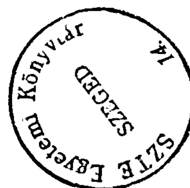
Table VIII Culture results on urethral discharge from 40 urethritis patients and seminal fluid from 30 healthy donors

Culture results	No. of urethritis patients	No. of healthy donors
Only aerobic bacteria	18	6
Only anaerobic bacteria	2	0
Aerobic and anaerobic bacteria	7	0
Mycoplasma hominis	0	0
Ureaplasma ureolyticum	3	0
Chlamydia trachomatis	3	0
Negative	7	24

The distribution of the aerobic and anaerobic isolates obtained from the urethritis patients is shown in Table IX.

Table IX Distribution of species isolated from urethral discharge of 40 urethritis patients

Species	No. of isolates
Gram-positive aerobes	
Streptococcus α-haemolyticus	5
Streptococcus agalactiae	2
Enterococcus faecalis	7
Staphylococcus spp.	2
Corynebacterium spp.	1
Gram-negative aerobes	
Escherichia coli	7
Klebsiella spp.	2
Enterobacter spp.	1
Proteus spp.	1
Haemophylus influenzae	1
Fungi	
Candida glabrata	1
Total aerobes	30
Gram-positive anaerobes	
Peptostreptococcus spp	8
Propionibacterium spp.	3
Eubacterium spp	2
Bifidobacterium spp.	2
Gram-negative anaerobes	
Prevotella spp.	1
Porphyromonas spp.	1
Veillonella spp.	3
Total anaerobes	20
Others	
Chlamydia trachomatis	3
Ureaplasma urealyticum	3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Typical aerobic uropathogens were isolated from 10 patients. Peptostreptococci were the most frequently isolated anaerobic bacteria. Thirty aerobic bacteria were isolated from 25 patients, and 20 anaerobic bacteria from 9 patients, 2.2 bacteria/positive patient.

The aerobic and anaerobic culture results on the seminal fluid of healthy donors revealed no anaerobic bacteria, and aerobic bacteria were present in only few cases (6 cases), with a low CFU/ml ($<10^2$).

Prostatitis is a difficult and puzzling syndrome, which rarely affects younger male adults, but is relatively frequent among men aged 30 years or more (50, 51). It is recognized that there are several distinct forms of the syndrome and successful treatment depends on a specific diagnostic procedure. Chronic bacterial prostatitis requires special bacteriological investigation by the Stamey test and microscopic evaluation of the presence of leukocytes in the specimen (52). It is well documented that streptococci and usual urinary pathogens such as *E. coli* and other members of the *Enterobacteriaceae* may be responsible for the chronic infection. However, in some cases, where ultrasonographic investigation of the prostate and the presence of leukocytes in the urine sample or in the EPS after prostatic massage suggest prolonged bacterial infection, the customary routine culture techniques may not reveal the pathogens. In some prostatitis cases, considered to be non-bacterial because of the negative culture results, it is difficult to know whether only improper laboratory methods are responsible for the diagnostic failures. In the present study, a careful culture technique was used to isolate aerobic, facultative anaerobic and strict anaerobic bacteria from the urethral sample and EPS of 169 patients with prolonged symptoms of chronic prostatitis, despite empiric quinolone therapy. The primary plates for anaerobic bacteria were incubated for a longer period (6 days) than usually applied in the routine procedures. From 56 patients, an average of 5.34 anaerobic bacteria were isolated. In 8 patients, the mixed anaerobic bacteria were accompanied by aerobic species. The low numbers of *S. epidermidis*, *S. α-haemolyticus* and *Corynebacterium* spp. can be considered contaminants derived from the skin flora in these cases. The lowest number of different anaerobic species was 3, whereas 5 patients had ≥8 different anaerobic species in their EPS after prostatic massage.

All patients who exhibited mixed anaerobic bacteria in their EPS had previously undergone several routine culture tests using the Stamey criteria, without any positive culture results because in most laboratories anaerobic culture methods are not routinely used for these patients. The role of anaerobic bacteria in chronic prostatitis has not yet been studied in detail.

Only a few reports have been published on attempts to isolate anaerobic bacteria from the prostatic fluid of patients with chronic bacterial or non-bacterial prostatitis (17, 19).

The normal flora of the male urethra and prostatic fluid of 46 healthy subjects was evaluated by Ambrose *et al.* (53). No anaerobes were recovered from the urethra and only 2 *Bacteroides* spp. were found in the prostatic fluid. On the other hand, Finegold *et al.* studied "urethral" urine (the first 10 to 20 ml of voided urine) in 17 subjects, and recovered anaerobes (together with aerobes) from 8 of these specimens. The colony counts of anaerobes were $\leq 10^4$/ml. The organisms recovered included anaerobic Gram-positive cocci and bacilli, as well as *Bacteroides* spp. (18). In our study, only 9 of 40 urethritis patients displayed anaerobic bacteria in the urethral sample (mostly *Peptostreptococci, Propionibacterium* and Gram-positive non-spore-forming bacilli). The average number of anaerobic species/positive patient was 2.2. Anaerobic bacteria were not isolated from the seminal fluid of 30 healthy donors.

Treatment of chronic prostatitis patients with positive culture results for anaerobes was carried out for 3 to 6 weeks with amoxicillin/clavulanic acid or clindamycin. This decreased or totally eliminated the complaints of the patients and the typical clinical symptoms of prostatitis, and the post-treatment cultures of the EPS obtained after prostatic massage were negative for anaerobes or only Gram-positive anaerobic bacteria such as peptostreptococci or *Propionibacteria* were grown in low number. In general, for chronic bacterial prostatitis patients a prolonged course of treatment, an increased dose of antibiotic, or both is suggested (17, 54). The most widely-used antibiotics are the fluoroquinolones because of their broad spectrum of action against Gram-positive and Gram-negative aerobic bacteria and their good penetration into the prostatic tissues (21, 55-57). In our cases, amoxicillin/clavulanic acid or clindamycin was used for the

prolonged treatment because of their good activity against anaerobic bacteria and their good penetration into the prostatic tissue (55).

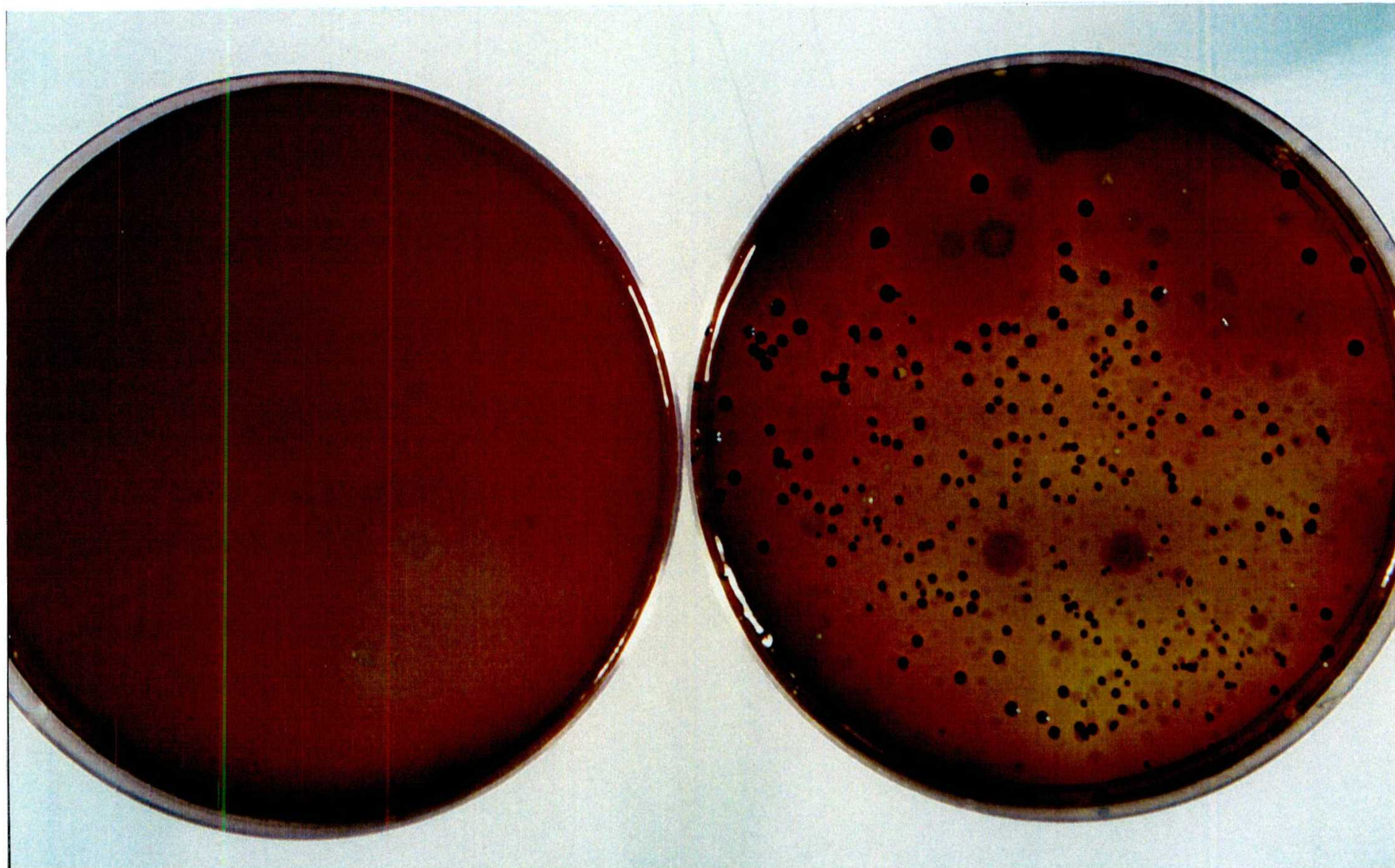
In this study, *B. ureolyticus* was isolated from mixed cultures from 50% of the positive patients (28 of 56 patients) with chronic prostatitis. *B. ureolyticus* is considered to be part of the normal flora in both the male and female genital tract, but it has also been isolated from mixed cultures of infections of virtually every organ system in men (58). It has previously been found to be associated with male non-gonococcal urethritis, where it was suggested that this organism may have a pathogenic role (59, 60). Eley *et al.* studied the pathogenicity of *B. ureolyticus* in animals and established that a pure culture of *B. ureolyticus* is capable of producing infection when it is injected subcutaneously in mice (61). The frequent isolation of *B. ureolyticus* together with other Gram-positive and Gram-negative anaerobes from mixed cultures among our patients indicated its possible pathogenic role in chronic bacterial prostatitis.

Our results suggest that anaerobic bacteria may be etiological agents in some cases of therapy-resistant, chronic bacterial prostatitis, and anaerobic culture methods involving prolonged incubation may be required for the laboratory diagnosis of chronic prostatitis.

3. Results of examination on biofilm flora of patients with oral cancer (papers VII, IX, X and XI)

Malodour and other infectious sequelae may increase the morbidity of patients with oral malignant neoplasias. It has been observed that poor oral hygiene increases the risk of oral cancer, but little attention appears to have been directed to the microflora of the lesion itself, or the relevance of that flora to overall patient morbidity. Our results shows that the carcinoma surface biofilm harbors increased levels of both aerobic and anaerobic bacteria. The flora of the biofilm on the carcinoma surface may differ totally from the flora on the normal mucosal surface of the same patients. The increased number of anaerobes and the more prevalent presence of Gram-negative anaerobic species may cause the unpleasant odour in these patients (Fig. 5).

Fig. 5
Difference between numbers of colonies on anaerobic blood agar (dilution 10^{-4})



healthy site tumor site

Main anaerobic species increased at tumor sites included *Prevotella* spp., *Porphyromonas* spp., *Fusobacterium* spp. and species belonging to the *B. ureolyticus* group (Table X).

Table X Distribution of anaerobic bacteria isolated from the surfaces of healthy and tumor sites in the oral cavity (21 patients)

Species	Tumor site	Healthy site
Gram-negative		
Fusobacterium spp.	14	1
Prevotella spp.	11	2
Porphyromonas spp.	9	0
Bacteroides ureolyticus/gracilis	8	2
Veillonella spp.	18	9
Capnocytophaga spp.	1	0
Gram-positive		
Actinomyces spp.	11	1
Clostridium spp.	5	1
Propionibacterium spp.	9	1
Peptostreptococcus spp.	30	26
Lactobacillus spp.	12	8
Streptococcus constellatus	1	1
Gemella morbillorum	1	3

There were no differences in the aerobic species between the healthy and tumor sites, but the CFU/ml was higher in the samples originating from tumors. In 4 of the 21 cases, we found *Candida albicans* at tumor sites and in 1 case at a healthy site (Table XI).

Table XI Distribution of aerobic bacteria isolated from the surfaces of healthy and tumor sites in the oral cavity (21 patient)

Species	Tumour site	Heathy site
Gram-negative		
Haemophilus influenzae	14	11
Haemophilus parainfluenzae	3	4
Haemophilus haemolyticus	1	1
Serratia liquefaciens	3	1
Klebsiella pneumoniae	2	0
Escherichia coli	1	1
Citrobacter freundii	1	0
Pseudomonas spp.	1	1
Branhamella catharralis	2	3
Neisseria spp.	13	20
Gram-positive		
Streptococcus β-haemolyticus	3	0
Staphylococcus aureus	2	1
Enterococcus faecalis	12	9
Streptococcus α-haemolyticus	33	33
Staphylococcus spp. (coagulase-negative)	15	15
Corynebacterium spp.	5	7
Fungi		
Candida albicans	4	1

Data relating to tumor and control sites were compared by the Wilcoxon Matched-Pairs Signed Ranks Test in aerobic and anaerobic cases. In both cases, the median values at tumor sites were higher than those at healthy sites. The differences were significant ($p = 0.0001$ for anaerobic bacteria, $p = 0.0008$ for aerobic bacteria). The significance level was set at $p < 0.01$.

Table XII lists the CFU/ml and the median values for aerobic and anaerobic bacteria.

Table XII Values of CFU/ml of aerobic and anaerobic bacteria

Sample		CFU/ml		
		Minimum	Maximum	Median
Aerobic	Tumor site	$2.5x10^5$	$1x10^9$	$3.45x10^7$
	Healthy site	$1x10^5$	$7x10^7$	$2x10^6$
Anaerobic	Tumor site	$1x10^2$	$1x10^9$	$7.5x10^7$
	Healthy site	$3x10^4$	$2x10^8$	$1.45x10^7$

This study revealed that, relative to the contiguous healthy oral mucosa, human oral carcinoma surface biofilms harbored significantly increased levels of both aerobes and anaerobes, including many species associated with oral infections, and with focal infection of oral origin. *Candida* spp. were observed at the lesion sites of only 5 of the 21 patients. These findings are very significant in relation to the morbidity of patients with such lesions, and not simply in terms of the oral hygiene level, or due to the immunosuppression caused by tumor treatment (chemotherapy). The finding of *Candida* species on the lesions in the present study indicates a need for their suppression, if present, before any tumor treatment is given, as severe complications can otherwise result (61). The compromised host response may partly explain the unfavorable microflora shifts observed in oral carcinoma surface biofilms. A major additional factor would logically seem to be the irregularity of the lesion surface, providing stagnant niches favoring microbial retention oral growth, especially of anaerobic organisms. The increases in variety and number of microniches relate therefore to the increased range and total count of microorganisms in cancer as compared to control sites.

4. Results on toxin production of *B. fragilis* (paper II)

Enterotoxigenic strains of *B. fragilis* have been demonstrated to be associated with diarrhoea in domestic animals and humans. The supernatants of *B. fragilis* strains which were considered to be ETBF induced cytotoxic changes on the HT-29 cell line which were clearly visible after 4 h of incubation (Fig. 6). Table XIII summarizes the data relating to the production of enterotoxin by the *B. fragilis* strains originating from different sources.

Table XIII Enterotoxin production by *B. fragilis* strains from different sources

Sources of strains	Numbers of strains		Percentage of
	tested	toxin-positive	toxin-positive
Faeces of patients with diarrhoea	40	16	40%
Faeces of healthy subjects	20	3	15%
Abscesses, etc.	74	15	20.3%

Among the 134 strains examined, 34 ETBF strains were found (25.3%). The cytotoxic titers were 1:4 or higher in all cases. Strains from extraintestinal infections tended to have higher cytotoxic titers than strains isolated from faeces (Table XIV).

Table XIV Toxin titers of different ETBF strains

Sources of strains	No. of strains	Titers			
		1:2	1:4	1:8	≥1:16
From infections	15	0	1	4	10
From faeces of patients with diarrhoea	16	0	5	6	5
From faeces of healthy subjects	3	0	0	3	0

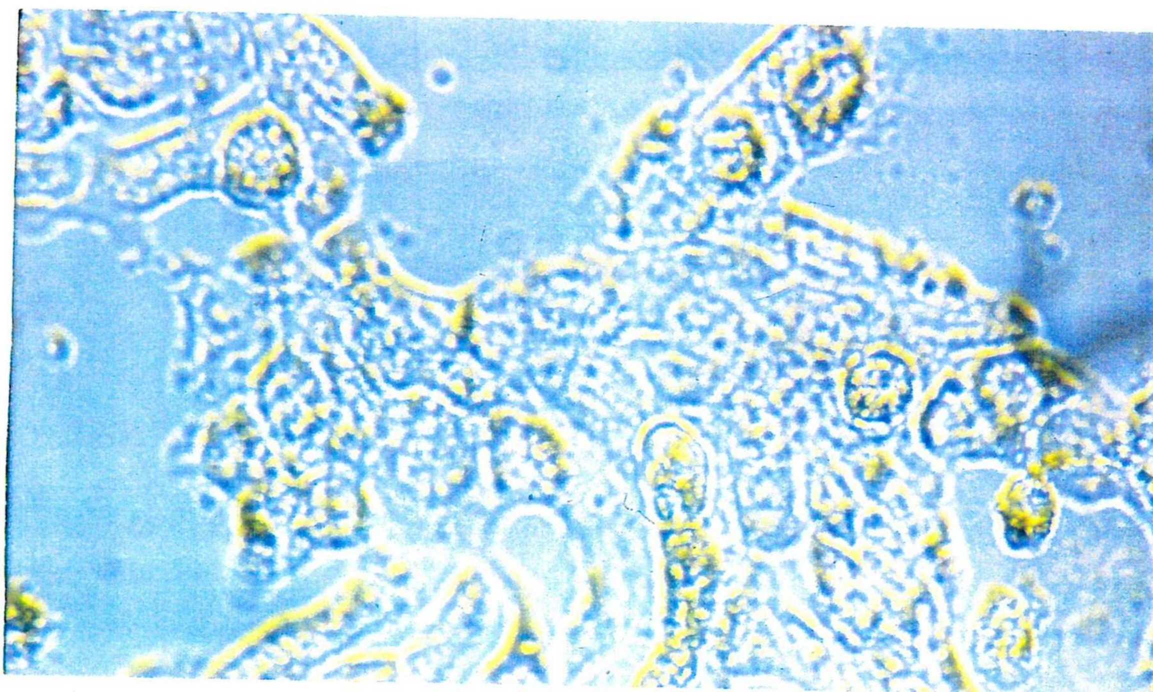
The incidence of ETBF strains was higher among the faecal isolates from patients with diarrhoea (40%) than among the strains originating from infectious processes (20.3%) or from the faeces of healthy individuals (15%) (Table XIV). During the direct detection of the toxin in the faecal filtrates, similar morphological changes in HT-29 cells were observed, but the toxic effects were detectable only at low dilutions (≤4). Of 50 faecal samples originating from patients with diarrhoea, 20 (40%) proved to be positive on the HT-29 cell line. The faecal filtrates did not induce cytotoxic changes in the McCoy, HeLa and Hep2 cells used in our laboratory for the detection of *Cl. difficile* toxin. The toxic effects of the *B. fragilis* culture supernatants and the faecal filtrates could not be neutralized by preincubation with *C. difficile* antitoxin. One faecal filtrate had a positive cytopathic effect on Vero cells. The 40 *B. fragilis* strains isolated from these faecal samples were also tested for the presence of enterotoxin: 16 (40%) exhibited a typical cytopathic effect (Fig. 6).

ETBF strains have been found in different parts of the world (USA, Italy, Poland, etc.) among *B. fragilis* strains originating from severe infections and faecal specimens (25, 67). ETBF strains were observed in faecal samples with a frequency of 1-19% (25, 26, 67), whereas among *B. fragilis* strains isolated from infectious processes the frequency of ETBF strains was 23-52% (66). The highest incidence was observed among isolates derived from blood cultures. A similar incidence of ETBF strains was detected among the Hungarian isolates (25.3%). However, 40% of *B. fragilis* strains isolated from diarrhoeal stool specimens of adults selected on the basis of their not containing other known pathogens proved to be ETBF, but the supernatants of the strains tended to exhibit lower toxin titers. Among extraintestinal isolates, the frequency of ETBF was 20.3%. No blood culture-derived *B. fragilis* was tested during this study.

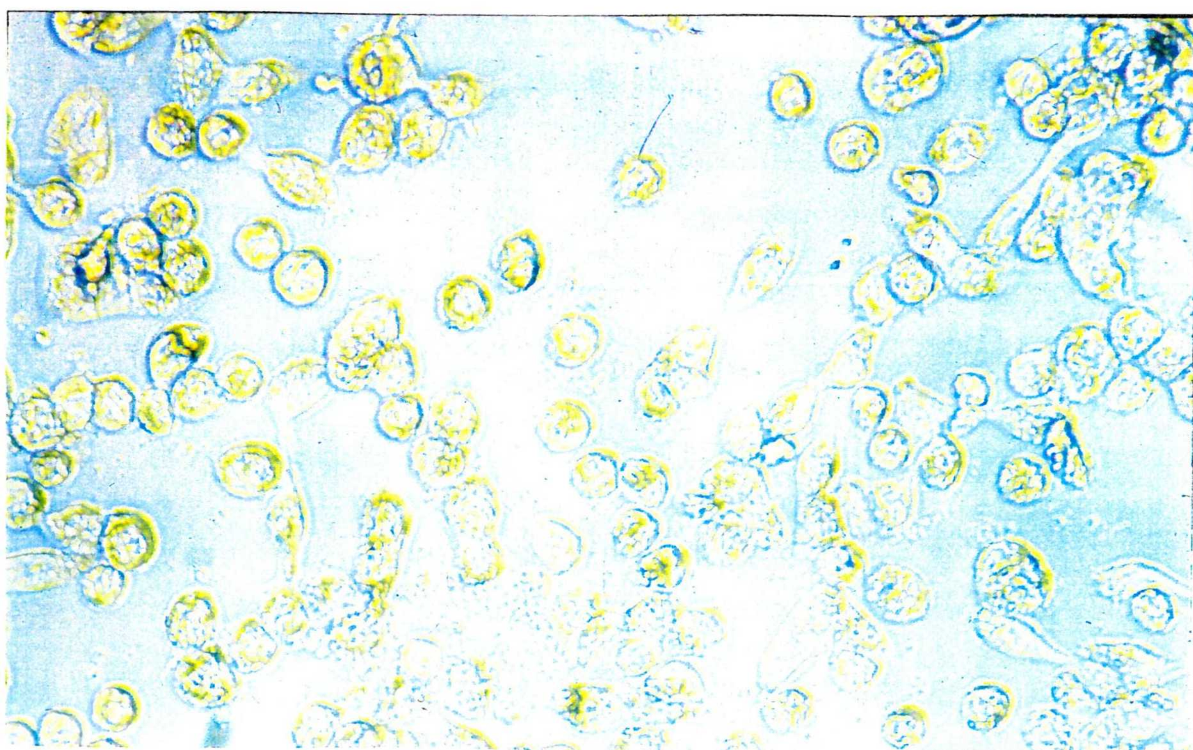
In our experience, the HT-29 cytotoxicity assay is easy to perform and reproducible. The cytotoxic effect is readily recognizable within 4 h and displays a clear-cut end-point. Approximately the same ratio of ETBF to total strains tested was found for our strains derived from faecal samples of healthy subjects and for the strains from extra-intestinal sources of Pantosti *et al.* (28). This might suggest that enterotoxin production is a neutral characteristic of these

Fig. 6. Effect of BHI culture filtrate of ETBF strains on HT-29 cells after exposure for 4 h

A: Untreated cells



B: Cytotoxic effect of ETBF isolated from abscess



strains, which does not provide any selective advantage over non-enterotoxigenic strains. However, a closer examination of the sources of ETBF demonstrates that an appreciable proportion of the high-titer toxin-producing strains were isolated from abscesses, where destructive processes are prominent. In terms of the pathogenesis of *B. fragilis*-induced infections, it is well established that the polysaccharide capsule of this species is essential in abscess formation, and hence is an important pathogenic factor. It has recently been confirmed that part of the DNA sequence of the enterotoxin gene of *B. fragilis* is homologous to that for the family of bacterial metalloproteases, which have emerged as an important virulence factor in a number of diverse pathogenic bacteria (68). In accord with this finding, the purified enterotoxin of *B. fragilis* exerts proteolytic activity if gelatin or actin is used as substrate *in vitro*. This metalloprotease activity of the enterotoxin of *B. fragilis* strains suggests a possible mechanism for enterotoxicity and a role in the invasiveness associated with *B. fragilis* soft-tissue infections.

5. Results of induction of cytokines by *Bacteroides* strains (papers VI and XII)

For the induction of both TNF and IL-6 10^8 CFU/ml bacterial cells were used according to the results obtained with the control *S. aureus* strain and the *B. fragilis* No. 7 (Fig. 7). All *Bacteroides* strains isolated from infectious processes or from stool specimens of healthy subjects induced TNF in mononuclear cells ($5x10^1$-$5x10^2$ U/ml). About 10 to 100 times more TNF was detected if whole blood was used, very probably because of the presence of serum factors. If strains were cultured in BHI broth containing horse serum, with few exceptions a one or more log higher amount of TNF was detected in the supernatant of the mononuclear cells as well as in the whole blood (Table XV). The induction of TNF was specific as anti-TNF monoclonal antibody inhibited its activity in the bioassay.

The IL-6 induction in mononuclear cells by *Bacteroides* strains belonging to different species was as effective as with the control *S. aureus* strain (Table XVI). The same tendencies were observed as for TNF induction if whole blood or strains cultured in BHI supplemented with horse serum were used. The activity in

Fig. 7
TNF levels measured by bioassay in the supernatants of human mononuclear cells ($5x10^6$/ml) stimulated with heat-killed *S. aureus* and *B. fragilis* (No. 7) at the concentrations indicated

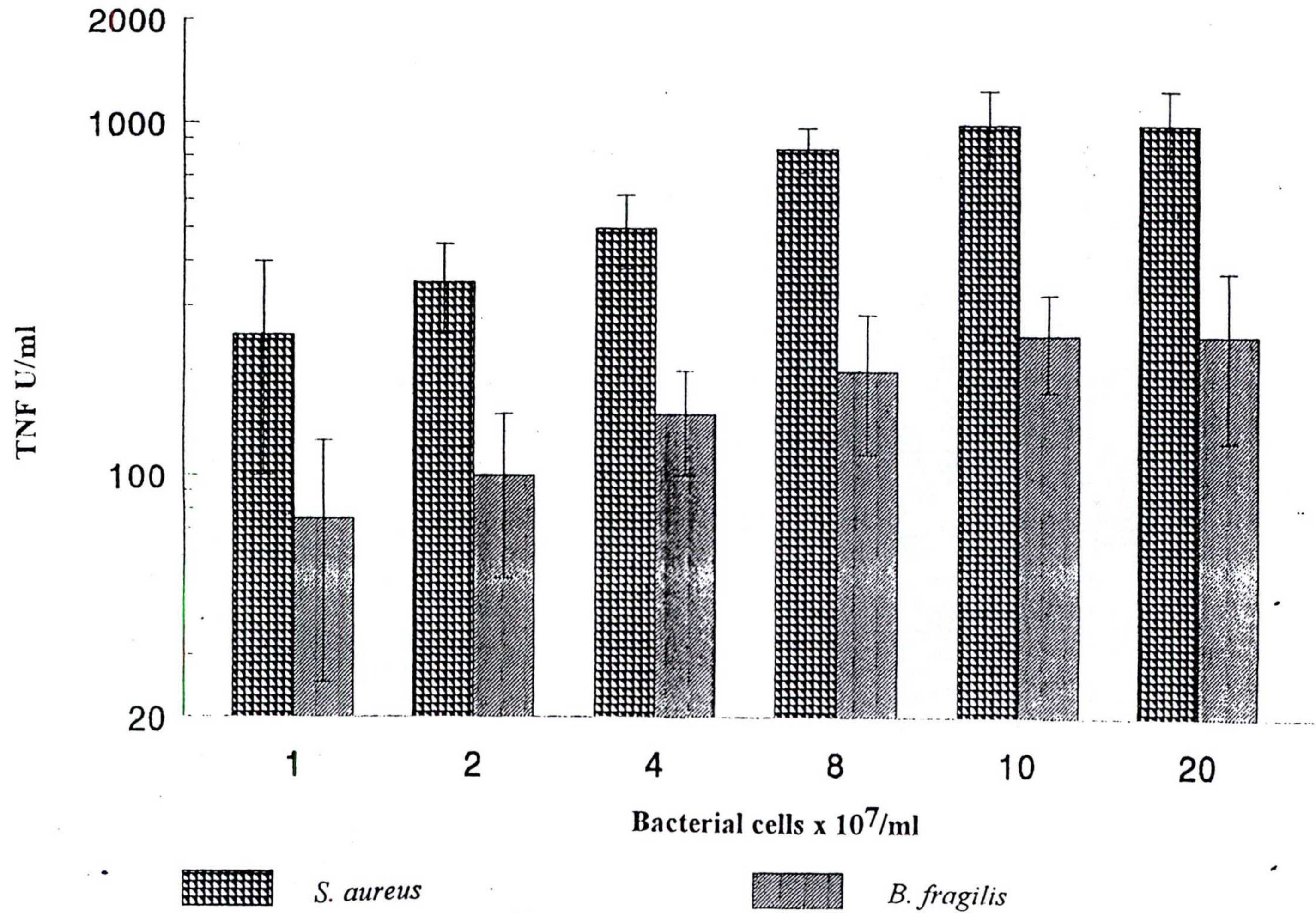


Table XV Induction of TNF by *Bacteroides* strains isolated from different sources

Strains		**Amount of TNF (U/ml) produced by**	
		mononuclear cells	**whole blood**
Strains from infections			
B. fragilis			
	1	$5x10^{1}$	$2x10^{2}$
	2	$5x10^{1}$ ($5x10^{2}$)*	$1x10^{2}$ ($5x10^{2}$)
	3	$2x10^{1}$ ($5x10^{2}$)	$1x10^{2}$ ($5x10^{2}$)
	4	$5x10^{1}$	$1x10^{3}$
	5	$1x10^{2}$ ($1x10^{2}$)	$5x10^{2}$ ($1x10^{3}$)
	6	$2x10^{2}$ ($5x10^{2}$)	$4x10^{2}$ ($5x10^{2}$)
	7	$5x10^{2}$ ($1x10^{3}$)	$2x10^{3}$ ($1x10^{4}$)
	8	$2x10^{2}$	$1x10^{3}$
	9	$1x10^{2}$ ($1x10^{3}$)	$5x10^{2}$ ($2x10^{3}$)
	10	$1x10^{2}$ ($4x10^{2}$)	$1x10^{2}$ ($5x10^{2}$)
B. ovatus	**1**	$1x10^{1}$	$2x10^{2}$
	2	$1x10^{2}$	$5x10^{2}$
	3	$7x10^{1}$	$2x10^{2}$
	4	$5x10^{1}$ ($5x10^{1}$)	$1x10^{2}$ ($2x10^{2}$)
	5	$5x10^{1}$ ($1x10^{2}$)	$7x10^{1}$ ($1x10^{3}$)
	6	$2x10^{2}$ ($5x10^{2}$)	$2x10^{2}$ ($4x10^{2}$)
B. vulgatus	**1**	$1x10^{1}$	$7x10^{1}$
	2	$5x10^{1}$	$2x10^{2}$
	3	$1x10^{2}$ ($2x10^{2}$)	$2x10^{2}$ ($1x10^{3}$)
	4	$5x10^{1}$ ($1x10^{2}$)	$1x10^{3}$ ($5x10^{3}$)
B. thetaiotaomicron	**1**	$2x10^{2}$	$8x10^{2}$
	2	$5x10^{1}$	$5x10^{2}$
	3	$1x10^{2}$	$8x10^{2}$
	4	$1x10^{2}$	$5x10^{2}$
	5	$5x10^{1}$	$1x10^{2}$
	6	$5x10^{2}$ ($5x10^{3}$)	$1x10^{3}$ ($5x10^{3}$)
B. splanchnicus	**1**	$1x10^{1}$	$2x10^{2}$
	2	$1x10^{2}$ ($7x10^{2}$)	$5x10^{2}$ ($2x10^{3}$)
Strains from normal feacal flora			
B.fragilis		1x102	2x102
B.vulgatus		1x101	1x102
B.caccae		1x101	1x102
B.merdae		2x101	5x102
***S.aureus* (Wood)**		2,5x102	1x103

* **Values in parentheses are the amounts of TNF induced by *Bacteroides* strains cultured in BHI broth supplemented with horse serum**

Table XVI Induction of IL-6 by *Bacteroides* strains isolated from different sources

Strains		**Amount of IL-6 (pg/ml) produced by**	
		mononuclear cells	**whole blood**
B. fragilis	**1**	**$1x10^4$**	**$1x10^4$**
	2	**$1x10^5$ ($1x10^5$)***	**$2x10^5$ ($5x10^5$)**
	3	**$1x10^5$ ($1x10^5$)**	**$1x10^5$ ($1x10^5$)**
	4	**$1x10^1$**	**$1x10^2$**
	5	**$3x10^5$ ($5x10^5$)**	**$5x10^5$ ($1x10^6$)**
	6	**$1x10^4$ ($1y10^5$)**	**$1x10^5$ ($1x10^5$)**
	7	**$5x10^5$ ($1x10^6$)**	**$1x10^6$ ($1x10^6$)**
	8	**$1x10^3$**	**$1x10^4$**
	9	**$1x10^4$ ($1x10^5$)**	**$2x10^4$ ($1x10^5$)**
	10	**$1x10^4$ ($5x10^4$)**	**$5x10^4$ ($1x10^6$)**
B. ovatus	**1**	**$5x10^3$**	**$5x10^3$**
	2	**$5x10^4$**	**$5x10^4$**
	3	**$5x10^4$**	**$1x10^5$**
	4	**$5x10^5$ ($5x10^5$)**	**$5x10^5$ ($1x10^6$)**
	5	**$3x10^5$ ($5x10^5$)**	**$5x10^5$ ($1x10^6$)**
	6	**$1x10^3$ ($5x10^3$)**	**$2x10^3$ ($5x10^3$)**
B. vulgatus	**1**	**$1x10^3$**	**$7x10^3$**
	2	**$1x10^5$**	**$2x10^5$**
	3	**$1x10^5$ ($2x10^6$)**	**$1x10^6$ ($1x10^6$)**
	4	**$2x10^3$ ($1x10^4$)**	**$1x10^4$ ($5x10^4$)**
B. thetaiotaomicron	**1**	**$1x10^5$**	**$1x10^5$**
	2	**$5x10^4$**	**$5x10^4$**
	3	**$1x10^5$**	**$1x10^5$**
	4	**$1x10^5$**	**$5x10^5$**
	5	**$1x10^4$**	**$1x10^5$**
	6	**$5x10^2$ ($5x10^2$)**	**$1x10^2$ ($1x10^3$)**
B. splanchnicus	**1**	**$2x10^4$**	**$3x10^4$**
	2	**$2x10^3$ ($1x10^4$)**	**$1x10^4$ ($5x10^4$)**
Strains from normal feacal flora			
B. fragilis		**$1x10^5$**	**$2x10^5$**
B. vulgatus		**$1x10^4$**	**$1x10^5$**
B. caccae		**$1x10^4$**	**$8x10^4$**
B. merdae		**$2x10^4$**	**$1x10^5$**
***S. aureus* (Wood)**		**$5x10^4$**	**$5x10^4$**

* **Value in parentheses are the amounts of IL-6 induced by *Bacteroides* strains cultured in BHI broth supplemented with horse serum**

the bioassay was specific as monoclonal antibody against IL-6 inhibited the reaction. Some *B. fragilis* isolates were more active inducers for both TNF and IL-6 than other isolates of the same species or other *Bacteroides* species. *B. fragilis* No. 7 was selected to isolate the LPS of the strain cultured in BHI and in BHI supplemented with 5% horse serum because the most expressed differences in the TNF and IL-6 induction was observed with this strain. Purified *B. fragilis* LPSs and L1 fractions of the purification process were also used at 10 μg/ml for induction of TNF and IL-6 in both mononuclear cells and whole blood (Table XVII). No differences were detected between the induction capacities of the LPSs of the *B. fragilis* cells cultured only in BHI or in BHI supplemented with horse serum. When LPS of *E. coli* O128 B12 was used, slightly more TNF was induced (< 2.5 times) and 10 times more IL-6 was detected. The inducing capacity of the L1 fractions of both cultures were lower in both assays than that of the purified LPS.

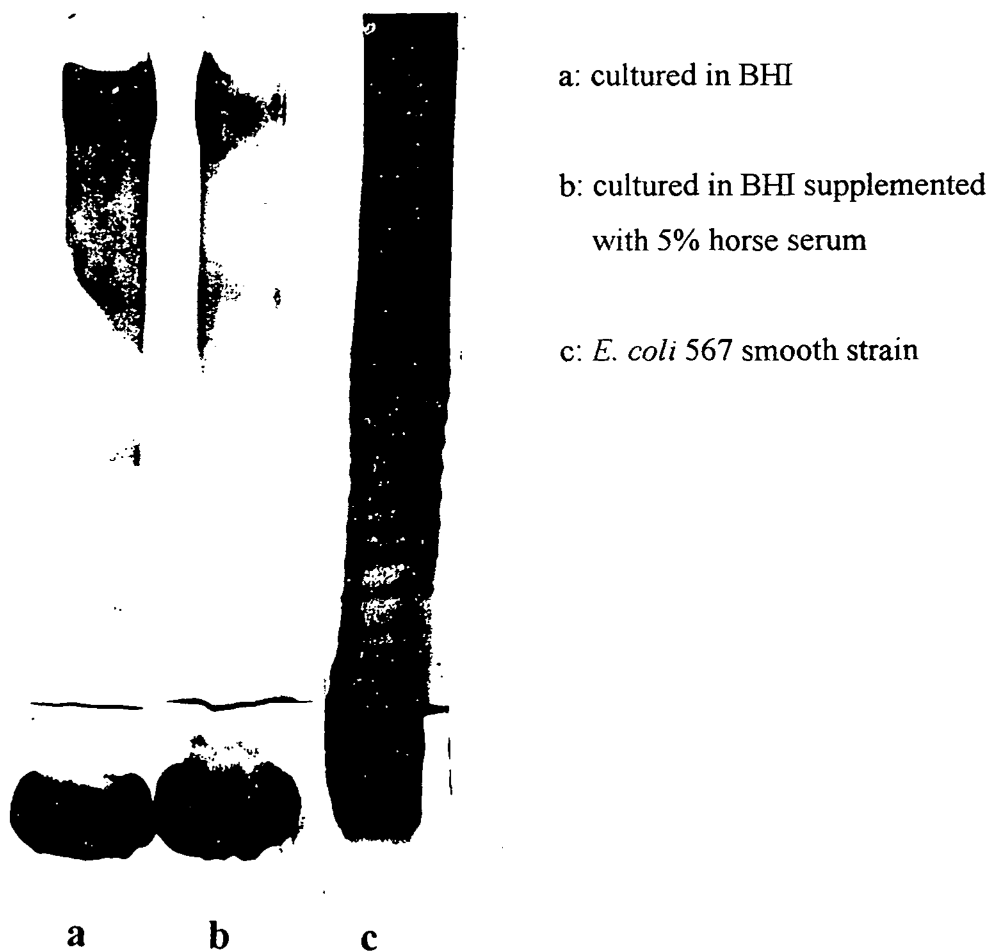
After silver staining, the LPS PAGE profiles of the *E. coli* (567) control strain displayed ladder patterns characteristic for the smooth LPS form. The profile of *B. fragilis* No. 7 extracts was rough with low molecular weights. We could not detect any difference between extracts of the same strain cultured in BHI or BHI supplemented with horse serum (Fig. 8). The L1 fraction contained sugars, proteins, nucleic acid and some LPS contamination.

During severe intra-abdominal or pelvic infections, a wide variety of Gram-negative anaerobic bacteria are involved besides *Enterobacteriaceae*: these are most frequently strains belonging in the genus *Bacteroides*. Together with IL-6, TNF is considered to be pivotal in the host response to bacterial invasion. These are produced in response to a bacterial insult, whether this is a whole organism, its components or its toxins. During shock-induced gut ischemia, the LPS of both the aerobic and anaerobic constituents of the bowel flora can translocate and induce the 'cascade of cytokines' . The role of *Bacteroides* strains, and especially *B. fragilis* (with more endotoxic rough LPS), which often outnumber *Enterobacteriacae* in the faeces and in the infectious processes with abscess formation, should not be overlooked from the point of view of inducing and/or modulating the host response in septic shock.

Table XVII Induction of TNF and IL-6 by LPS of *B. fragilis* (No. 7) cultured with or without serum

	Amount of			
	TNF (U/ml)		IL-6 (pg/ml)	
	MN cells	whole blood	MN cells	whole blood
***B. fragilis* LPS**				
cultured in BHI	10^2	$5x10^2$	10^5	10^5
cultured in BHI+s	10^2	$5x10^2$	10^5	10^5
Its L1 fraction				
cultured in BHI	10^2	$2x10^2$	10^4	10^4
cultured in BHI+s	$5x10^1$	$2x10^2$	10^4	10^4
***E. coli* LPS**	$2,5x10^2$	$7,5x10^2$	10^6	10^6

Fig. 8 LPS PAGE profile of *Bacteroides fragilis* No. 7



Conclusions

1. The adherence of *Bacteroides* species to fibronectin and to other ECM proteins is not necessarily connected with their pathogenicity, but may be the critical step for tissue invasion, especially if the adherence event induces other reactions possibly leading to the degradation of basement membranes.

2. Both molecular and specialized culture findings designed to detect fastidious and difficult-to-culture bacteria in prostatic tissue and fluids. This findings point to a possible etiologic role for these microorganisms in chronic idiopathic prostatitis.. Our results suggest that anaerobic bacteria may be etiological agents in some cases of therapy-resistant, chronic bacterial prostatitis, and anaerobic culture methods involving prolonged incubation may be required for the laboratory diagnosis of chronic prostatitis.

3. The cancer lesion itself may greatly increase the local and systemic infection risk to oral cancer patients, before, during and after specific tumor treatment. The findings indicate that, in addition to any other oral focus, the lesion itself when ulcerated should receive direct antimicrobial treatment so as to reduce patient morbidity.

4. Enterotoxin production is a neutral characteristic of *Bacteroides* strains, which does not provide any selective advantage over non-enterotoxigenic strains. However, a closer examination of the sources of ETBF demonstrates that an appreciable proportion of the high-titer toxin-producing strains were isolated from abscesses, where destructive processes are prominent.

5. All *Bacteroides* strains isolated from infectious processes or from stool specimens of healthy subjects induced TNF and IL-6 in mononuclear cells. These data support the opinion, that the lipopolysaccharide of *Bacteroides* strains, which outnumber aerobes in the gut flora and are involved in many serious infections, may play an important role in inducing the cascade of cytokines.

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Acknowledgments

I wish to express my special thanks to all those without whom this task would never have been fulfilled

My thanks are due to **Professor Elizabeth Nagy**, Head of the Department of Clinical Microbiology, for her untiring efforts and constant enthusiasm and professionalism in her supervision of this study.

I am very grateful to **Professor Torkel Wadström,** Head of the Department of Medical Microbiology of Lund University, for sharing with me his vast knowledge on ECM and protein chemistry.

Special thanks are due to **Dr Yvett Mándi** for her help in the TNF study, and to **Dr Béla Kocsis** for the isolation of LPS.

I wish to thank the **members of the staff at the Department of Clinical Microbiology** for their wide-ranging cooperation and for creating a peaceful working atmosphere.

I wish to express my appreciation to **Dr Katalin Nagy** and **Dr László Török** for their knowledge, advice and handling of the clinical part of these studies.

I am very grateful to **Dr Tibor Nyári** for his help in the statistical analysis of the results.