THE ROLE OF MESOTHELIAL CELLS IN THE HOST DEFENCE AGAINST BACTERIA

IN VITRO STUDIES OF PERITONITIS

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DE ROL VAN MESOTHEELCELLEN IN HET AFWEERSYSTEEM TEGEN BACTERIËN

PERITONITIS EXPERIMENTEN *IN VITRO*

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Cover: An electron micrograph shows neutrophilic granulocytes crossing a monolayer of cultured mesothelial cells

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ABBREVATIONS

AD	actinomycin D
CA 125	cancer antigen 125
CAPD	continous ambulatory peritoneal dialysis
FMLP	formyl-methionyl-leucyl-phenylalanine
HSA	human serum albumine
ICAM-1	intercellular adhesion molecule-1
IL-1	interleukin-1
IL-8	interleukin-8
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MAb	monoclonal antibody
MC	mesothelial cell(s)
MCP-1	monocyte chemoattractant protein-1
MFI	mean fluorescense activity
PAF	platelet activating factor
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
PMA	phorbol myristate acetate
PMN	polymorphonuclear leukocytes
SEP	sclerosing encapsulated peritonitis
sLe^x	sialyl-Lewis-X
TGF-β	transforming growth factor-β
TNF	tumor necrosis factor
VCAM-1	vascular adhesion molecule-1
VLA-4	very late antigen-4

CONTENTS

1

INTRODUCTION

INTRODUCTION

1. The Peritoneum

The peritoneum is a serous membrane that lines both the intra-abdominal wall and the viscera contained within the peritoneal cavity. Etymologically, 'peritoneum' means 'wrapped tightly around.'[1] The first description of the structure and function of the human peritoneum was given by James Doughlas in 1730.[2] He observed that it was 'everywhere smooth and even and lubricated by a fluid in order to preserve it from those inconveniences which otherwise would have followed from its continual attrition with other viscera.'

Human peritoneum covers roughly the same surface area as the skin, approximately 2 m^2 in the adult.[1] The parietal peritoneum covering the abdominal wall constitutes about 10%, while the peritoneum covering the visceral organs, constitutes about 90% of this surface area. In males the peritoneum is completely closed, but in females it is open at the site of the uterine tubes, where the tubal peritoneum meets the non-peritonized fimbriated mucosa of the Fallopian tube.

The peritoneum is a derivative of the embryological mesoderm and belongs to a distinct family of body tissues that share the same cellular composition and exhibit an identical histological architecture known as the serosa. The basic structure of the peritoneum appears to be rather simple; it is composed of a monolayer of mesothelial cells separated from the capillaries by a thin layer of loose connective interstitial tissue.

2. Mesothelial Cells

In 'en face' preparations the monolayer of mesothelial cells shows a mosaic of polygonal or 'cobble stone' cells (Figure 1).[3, 4] Mesothelium usually appears as a continuous cellular surface that is covered by numerous microvilli (Figure 2). In 1954 Odor was the first to describe the presence of microvilli covering the free surface of mesothelial cells by using transmission electron microscopy.[5] The functional significance of these microvilli is not clear. Odor proposed that these structures increase the mesothelial surface area, thereby facilitating the exchange of fluids and solutes between cells and the body cavity. Later, it was certified by transmission electron microscopy that the microvilli are cytoplasmic prolongations[6] which concurs with Odor's ideas.

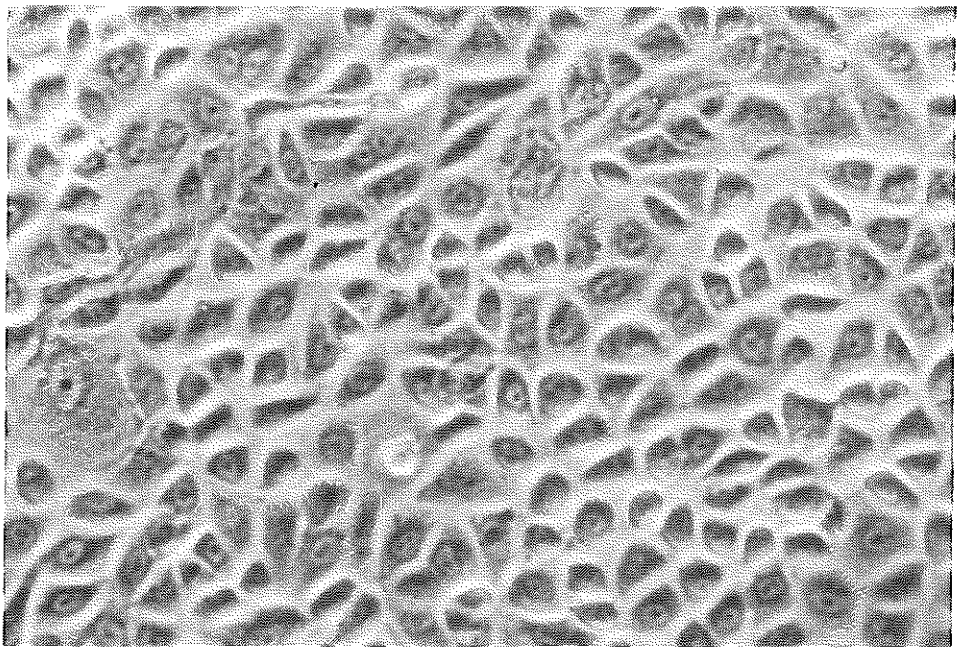

Figure 1. A monolayer of cultured human mesothelial cells (×400).

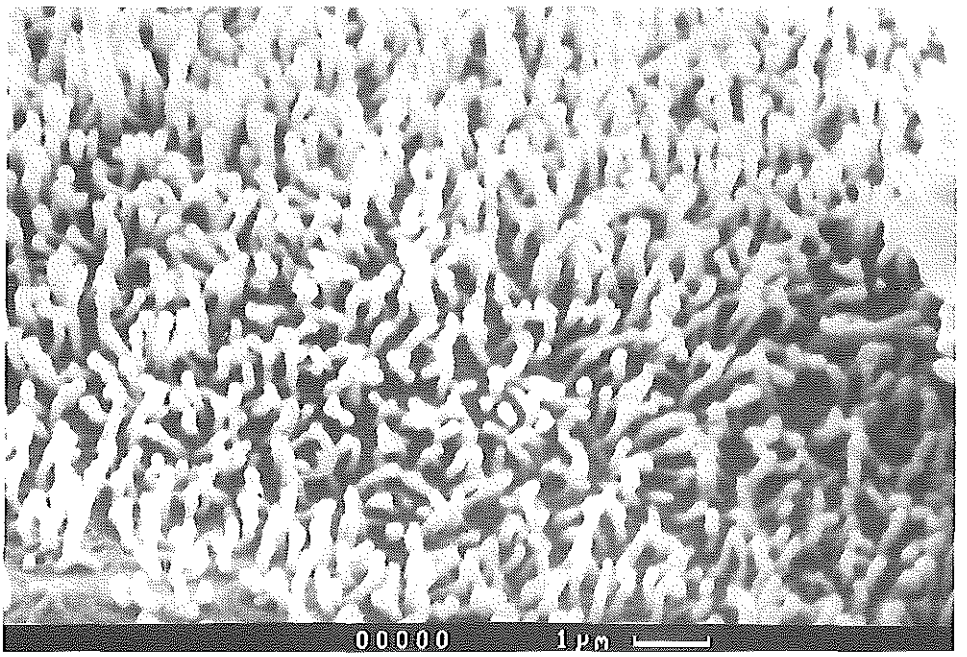

Figure 2. An electron micrograph shows a cultured human mesothelial cell covered with microvilli

A higher density of microvilli is present on those parts of the peritoneum that cover very mobile organs[6] which supports the hypothesis that microvilli, together with a serous exudate of the mesothelium, protect the mesothelium against friction and facilitate movement. In contrast, the mesothelium of fish is devoid of microvilli, which may be explained by the reduced exposure to frictional contact between peritoneal surfaces in fish compared to that experienced in land-based animals.[7]

The nucleus of the mesothelial cell has an oval or reniform shape and is usually located in the central region. Evidence that mesothelium is capable of secretory activity was provided by the detection of large nuclei in mesothelial cells, showing predominance of euchromatin over heterochromatin together with two prominent nucleoli.[8] Its secretory activity is further reflected by the abundance of rough endoplasmic reticulum and the prominence of well-developed Golgi complexes.[8]

Mesothelial intercellular junctions have been widely investigated.[9-11] They consist of tight junctions that form an anatomical barrier capable of restricting the passage of relatively small molecules from the capillaries into the peritoneal cavity.[12] Under normal circumstances, mesothelium acts as a semi-permeable membrane. The application of this membrane function in peritoneal dialysis was first described in 1962.[13] Peritoneal dialysis is now a major form of therapy for the treatment of acute and chronic renal insufficiency.

3. Peritonitis

Bacterial peritonitis is usually a polymicrobial infection, due to a perforation of the gastrointestinal tract.[14] The site of perforation determines which bacteria will be present in the abdominal cavity. Normally, the stomach and duodenum contain very few viable bacteria, owing to its gastric acidity; consequently, perforation of gastric or duodenal ulcers often initially causes a chemical, non-bacterial peritonitis. Going down the gastrointestinal tract, the micro-flora gradually changes to that of the colon. Peritonitis due to perforation at this level involves a microbiological spectrum of many different species in which anaerobes outnumber the aerobic species in a ratio of about 1000:1.[15]

Spontaneous bacterial peritonitis (SBP) is defined as bacterial infection of ascitic fluid without any identifiable intra-abdominal source,[16] i.e. without a surgically treatable source. Because most bacteria causing SBP are gut-derived, it is thought that enteric bacteria regularly translocate and enter the ascitic fluid, but that in the majority of such events these bacteria are cleared by

local host defence mechanisms.[17] Indeed, several studies have shown that patients who develop SBP have low chemoattractant and opsonic activities in their ascitic fluid.[18,19] SBP frequently occurs in patients with ascites (25%)[20] and cirrhosis (10%).[21]

In most cases, the diagnosis of peritonitis is straight-forward. Fever, leukocytosis, abdominal pain and tenderness are commonly seen. However, in elderly patients, patients receiving steroid therapy, and especially in postoperative patients, the signs and symptoms are often masked.[22] In contrast to 'surgical' peritonitis , the onset of continuous ambulatory peritoneal dialysis (CAPD)-associated peritonitis is often accompanied by only mild symptoms of peritoneal irritation.[23] Peritonitis is the most important complication in CAPD therapy, with an overall incidence of approximately 0.8 episodes per year.[24] In most cases the diagnosis of peritonitis in CAPD is simple. The usual presenting feature is a cloudy effluent, caused by the rapid rise in the number of neutrophils within the peritoneal cavity in the presence of infection.[25, 26] The bacteria that cause CAPD-associated peritonitis are usually derived from the skin (predominantly *staphylococci*) and enter the peritoneal cavity via the percutanous catheter.

Peritonitis of infectious origin is associated with the formation of intra-abdominal adhesions, a rapid process in which a fibrous network begins to develop within 10 minutes after trauma that is fully developed after 2 hours.[27, 28] These early fibrous attachments can either be absorbed completely or else become invaded by fibroblasts, which remodel it into fibrous adhesions. The formation of intra-abdominal adhesions may have important consequences: it causes 15-20% of female infertility,[29] and more than 30% of small bowel obstructions in developed countries.[30, 31] Sclerosing encapsulated peritonitis (SEP), an extremely severe form of adhesion formation, was first described by Slingeneyer et al.[32] It consists of the alteration of mesothelium into a thickened, leathery, fibroconnective sheath. It is usually observed after slowly resolving, massive intra-abdominal infection and frequently results in bowel obstructions and loss of ultrafiltration in CAPD patients.[33] The etiology of SEP has not been elucidated. However, certain lavage fluids used in surgery and substances used as buffer in dialysate fluids are associated with a higher prevalence of SEP.[34, 35]

Peritoneum plays an important role in the prevention of adhesion formation by its increased fibrinolytic activity after the formation of fibrous tissue. In 1963, Benzer et al. first demonstrated the fibrinolytic activity of human peritoneum.[36] Later, Raftery found that this activity was present mainly in the mesothelium.[37] However, the fibrinolytic potential of the mesothelium is strongly reduced after

experimental trauma of the mesothelium: this depression is seen immediately after injury and its degree varies, being the most dramatic after ischemic injury.[38, 39] Plasminogen activator inhibitor-1 (PAI-1), an important inhibitor of fibrinolysis,[40] seems to play an essential role in this reduction, resulting in persistence of intra-abdominal fibrin with subsequent adhesion formation.[41] Mesothelial cells are capable of PAI-1 synthesis; increased PAI-1 activity was found in activated cultured human mesothelium[42] and in biopsies of inflamed peritoneum.[41, 43] Recently, van Goor et al. found not only an increased fibrinolytic inhibitor (PAI-1) but also, in contrast with the classical concept, an increased fibrinolytic activator (tissue plasminogen activator (t-PA); also mesothelial derived[42]) in the abdominal cavity of patients with peritonitis. Further investigations will be necessary to delineate the trigger for disbalance of the fibrinolytic capacity of mesothelium during peritonitis.

Mesothelial damage and repair

Loss of mesothelial tissue has been described as a consequence of cell distress after episodes of peritonitis.[45, 46] Peritoneal healing after peritonitis differs distinctly from healing in other tissues; the healing time, which is usually very rapid (5-8 days), is independent of the initial size of the defect.[47, 48] Thus, peritoneal defects are not only repaired by centripetal movement from the wound margins, but also by other repair mechanisms. Several hypotheses have been proposed to explain peritoneal healing:

1. Mesothelial cells migrate inward from the wound margins.[49]
2. Mature mesothelial cells from intact peritoneum exfoliate, resettle and, thus, repopulate the defect.[50]
3. Serosal cells floating in the peritoneal fluid settle on the injured surface.[51]
4. Serosal connective tissue cells transform into mesothelial cells.[52,53]
5. Reparative cells originate from bone marrow.[54]

The exact nature of the repair mechanism remains controversial, but it seems quite possible that several of the above-mentioned processes act simultaneously.[48, 55] In 1990, Di Paolo et al. described a possible treatment for the mesothelial cell loss after severe peritonitis: autologous re-implant in CAPD patients of peritoneal mesothelium that had previously been removed, cultured and stored frozen.[56, 57] In their study morphological signs of repopulation were evident at 3 and 6 days after implantation.

4. New Approaches

The mortality rate of 35%[58] associated with severe surgical and other types of peritonitis has not improved dramatically in the last 50 years, despite the availability of powerful broad-spectrum antibiotics, intensive care units, and

radical approaches to eliminate the bacterial inoculum from the abdominal cavity.[59] An overaggressive systemic inflammatory response of patients with peritonitis often results in a generalized micro-circulatory failure, fluid sequestration, and the development of multiple organ failure; the latter event is currently the major cause of death following intra-abdominal infections.[60] An in-depth understanding of the host defence during peritonitis, therefore, is necessary to develop new forms of therapy that can be used as an adjunct to the existing surgical techniques and antibiotic treatment strategies.

5. Immunological Aspects

Normally, the peritoneal cavity contains less than 50 ml of fluid, with a low number of white blood cells consisting of approximately 90% macrophages, 5 to 10% lymphocytes and fewer than 5% polymorphonuclear leukocytes (PMN).[61] However, during the onset of peritonitis a massive influx of neutrophils into the peritoneal fluid is observed as an early response to bacterial invasion. Within 24 hours this is followed by the migration of large numbers of monocytes.[62, 63] In peritonitis, the PMN and monocytes not only have to migrate across endothelial cells when leaving the circulation, but also across the mesothelial monolayer before they can enter the peritoneal cavity (Figure 3).

Leukocyte influx

From previous studies it is known that leukocyte influx into the tissues is a well-coordinated process. Leukocyte migration across endothelial cells has been studied in detail; it plays a role in all inflammatory processes throughout the human body. The adherence of leukocytes to endothelial cells and subsequent migration across the endothelial barrier into the adjacent tissue takes place through several specific adhesion receptors on both cell types.

Under resting conditions, neutrophils do not attach to other cells, but require triggering by external stimuli to become adhesive. The adhesion of neutrophils to endothelial cells is thought to be a two-step process.[64] In the first step, leukocyte selectin (L-selectin) on the neutrophils transiently binds to the sialyl-Lewis-X (sLe^x) carbohydrate structures on the endothelial cells (Table 1). In addition, endothelial selectin (E-selectin) binds in a similar fashion to sLe^x structures on the PMN (Table 1).[65] These bonds are relatively weak and cause the initial 'rolling' of neutrophils along the endothelium. E-selectin is not expressed on resting endothelial cells, but does appear on endothelial cells approximately 1 hour after activation with tumor necrosis factor (TNF), interleukin-1 (IL-1) or lipopolysaccharide (LPS). E-selectin expression on endothelial cells reaches its peak after 4-8 hours and is back to baseline by 24-

48 hours *in vitro*.[66] Binding of sLe^x ligands to L- and E-selectin reduces the velocity of the PMN and precedes strong adhesion and transmigration.[64, 67]

Table 1. Endothelial adhesion molecules and their counterstructures.

Endothelial adhesion molecule	*Counterstructure*
$S\text{-}Le^x$ moieties	L-selectin (*neutrophils*)
E-selectin	$S\text{-}Le^x$ moieties (*neutrophils*)
ICAM-1	CR3 (CD11/CD18; *neutrophils, monocytes*)
VCAM-1	VLA-4 (*monocytes*)

The second step of 'firm' adherence of neutrophils to endothelial cells is dependent on the family of β_2 integrin proteins on the PMN, of which CR3 (CD11b/CD18) is known to be the most important leukocyte adhesion molecule involved.[68, 69] The adhesion protein 'intercellular adhesion molecule-1' (ICAM-1)[70] found on activated endothelium is the best-known counter structure for CR3 (Table 1),[71, 72] but others may exist as well. From previous studies with endothelium it has become clear that an increase in the affinity of CR3 on PMN for its ligands on endothelial cells is an essential step in the adherence of PMN to endothelium.[67, 73] This increase in CR3 affinity can be induced by interaction between E-selectin, present on activated endothelial cells, and sLe^x-bearing glycoproteins on the PMN.[67] It is also known that activation of CR3 may occur by soluble stimuli (e.g. chemoattractants), independent of E-selectin ligand binding.[64]

In contrast to neutrophils, monocytes adhere avidly to resting endothelial cells.[74] This is probably an *in vitro* artefact caused by the purification of the monocytes.[75] Multiple ligand-receptor systems are involved in monocyte adherence. Monocytes can adhere not only to E-selectin and ICAM-1 on endothelial cells, as described for neutrophils, but can also bind to 'vascular cell adhesion molecule-1' (VCAM-1). VCAM-1 becomes increasingly expressed upon activation of endothelial cells.[76] The leukocyte receptor for VCAM-1 is 'very late antigen-4' (VLA-4; Table 1),[77] a member of the β_1 integrin family, which is expressed on monocytes but is absent on neutrophils.[78]

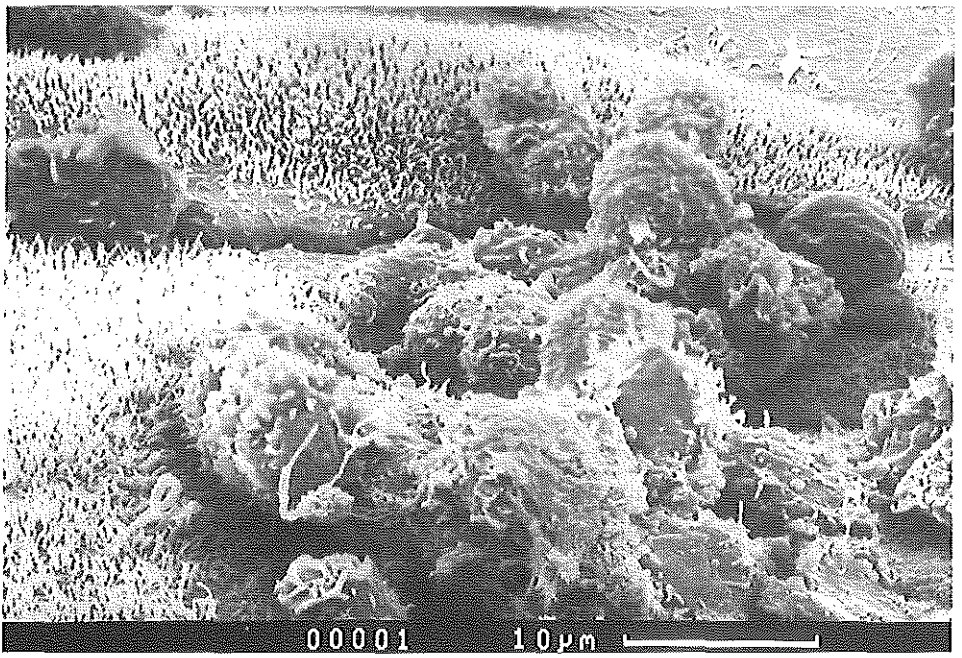

Figure 3. An electron micrograph shows neutrophilic granulocytes crossing a monolayer of cultured mesothelial cells

Monocytes generally exhibit a high rate of spontaneous migration across resting endothelial monolayers,[76, 79] which may become even higher after activation of the endothelial cells with IL-1. The inhibitory effects of anti-E-selectin and CD18 mAbs on monocyte adherence to and migration across activated endothelial cell monolayers is further enhanced when these mAbs are used in combination with an antibody to VLA-4.[76] Incubation of the monocytes with anti-VLA-4 mAb alone has no inhibitory effect, indicating that the VCAM-1/VLA-4 interactions alone are not sufficient for either monocyte adherence or migration.

Chemoattractants

Leukocyte migration towards inflammatory tissue is guided by locally produced chemoattractants. Several neutrophil chemoattractants have been characterized; the anaphylatoxin C5a,[80] formylmethionyl peptides of bacterial origin (FMLP),[81] leukotriene B_4 (LTB_4) of neutrophil origin, transforming growth factor-β (TGF-β),[82] platelet activating factor (PAF),[83] and interleukin-8 (IL-8; Figure 4).[84] Previous *in vitro* studies have shown that activated endothelial cells synthesize PAF[85] and IL-8.[86] Although these cytokines do not alter PMN adherence, they do have an additive stimulating effect on PMN migration across activated endothelial monolayers.[67, 87]

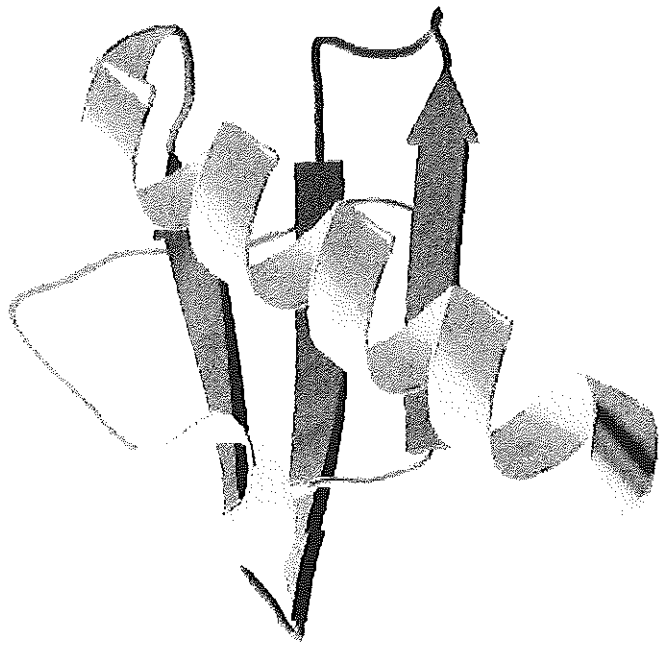

Figure 4. Human interleukin-8 (helix/sheet) (source: Internet; Cytokines Web)

IL-8 can be generated by a variety of cells[88] and is a strong and specific chemoattractant for neutrophils. It has been postulated that IL-8 forms a chemotactic gradient of matrix-associated IL-8 along endothelium, which might be required for neutrophil transmigration.[89,90] Once IL-8 has been released into the blood stream, it is bound and neutralized by red blood cells, which may thus provide a line of defence against systemic activation and against chemotactic disorientation of neutrophils by maintaining the chemotactic gradient.[91]

Neutrophils have the capacity to produce IL-8 themselves, in particular as a consequence of phagocytosis,[92, 93] and thus can in this way intensify the recruitment of new defence cells themselves. The long duration of IL-8 action *in vivo* is probably due to the remarkable resistance of IL-8 to enzymatic inactivation and denaturation.[94]

Jonjić et al.[95] and Goodman et al.[96] in 1992 described the secretion of IL-8 by cultured peritoneal and pleural mesothelium, respectively. This 'spontaneous' secretion of IL-8 could be augmented by IL-1, TNF, or interferon-γ. Later, an

increased concentration of IL-8 in drain dialysate during CAPD peritonitis was described by Lin et al.[97]

IL-8 belongs, together with 'monocyte chemotactic protein'-1 (MCP-1), to a family of small proteins (8-10 kDa) with four conserved cysteine residues. A very distinct difference between the IL-8 (C-X-C) and MCP-1 (C-C) subgroups is the presence or absence of an extra amino-acid between the first 2 cysteines. This difference is probably in part responsible for the binding specificity to neutrophils *versus* monocytes. Minor modifications in the amino-acid sequence of MCP-1 can change it into a ligand for the IL-8 receptor.[98] Systemic action of IL-8 and MCP-1 under physiological conditions is inhibited by serum IgG auto-antibodies against these cytokines.[99, 100]

Generally, monocytes are described as 'slow travellers' towards inflammatory tissue when compared with neutrophils.[61, 62, 101-103] However, many different chemoattractants for monocytes have been identified.[104-106] Mesothelium also synthesizes monocyte chemoattractants. Gerwin et al.[107] and Jonjić et al.[95] have described the secretion of TGF-β and the expression of MCP-1 mRNA by activated human mesothelial cells, respectively.

6. Neutrophil: friend or foe?

Infiltration of neutrophils into tissues plays an important role in the initial host defence against micro-organisms. This is exemplified by patients with the so-called 'leukocyte adhesion deficiency' (LAD). LAD patients lack the ability to invoke a normal neutrophil influx into infected or injured tissues. LAD is an autosomal recessive inherited disorder,[108] caused by a partial or total deficiency of β_2 integrins on the surface of leukocytes.[109] The degree of clinical symptoms is directly proportional to the degree of the deficiency.[110] Patients with LAD suffer from recurrent infections with impaired pus formation and defective wound healing. They may also display other remarkable features, such as delayed detachment of the umbilical stump, necrotic soft tissue lesions, and granulocytosis.[111] Interestingly, patients with LAD are not often victim of pneumonia or intra-abdominal sepsis.[112]

During inflammation, neutrophils release a remarkable arsenal of toxic substances, including oxygen radicals and proteolytic enzymes[113, 114] that can eliminate most of the offending pathogens. Although this neutrophil response is rapid and effective, it also has one major drawback: neutrophil oxidants are seen as the final mediators of tissue damage.[115] In recent years, the awareness of the neutrophils' capacity for an exaggerated autotoxic inflammatory response

has increased, and so has the desire to develop therapeutic immuno-modulating interventions that may attenuate these cells' unwanted destructive side effects.

7. Immuno-modulation

Interference with normal neutrophil host-defence functions has primarily been studied in animal models of the inflammatory response. mAb against ICAM-1 have proved successful in the therapy for postburn injury[116] and have reduced the neurological damage in a rabbit stroke model.[117] CD11/CD18 mAb against β_2 integrins *in vivo* strongly reduce formation of localized edema, tissue injury, and mortality in experimental bacterial meningitis.[118-120] Although CD11/CD18 mAb helped to maintain the blood-brain barrier, they also reduced the penetration of antibiotics into the cerebrospinal fluid. However, the rate of bacterial killing in the cerebrospinal fluid was unaffected and survival was improved. After massive inoculation of subcutaneous *Staphylococcus aureus* (>10^9CFU) in rabbits, the CD18 mAb-treated animals developed larger abscesses than controls.[112] Whether these findings are clinically important needs to be established. However, the protective effect of CD11/CD18 mAb correlates well with the prevention of phagocyte influx and hence underscores the role of the inflammatory infiltrate in these pathological processes.

Immunomodulating therapy in sepsis is more complex, due to the multitude of mediators present. While mortality and tissue injury are substantially reduced by mAb CD11/CD18 in hemorrhagic shock,[121,122] the value of CD18 mAb[122, 123] and anti-TNF therapy[124, 125] are less clear in sepsis. Anti-TNF therapy is not beneficial in experimental peritonitis in rats,[126] and neither is it successful when given after the full syndrome of septic shock has already developed.

Neutrophil influx into inflammatory tissue is a multi-step process; in addition to the modulation of the influx with mAb against TNF or mAb against the adhesion receptors CD11/CD18 and ICAM-1, interference can also be established by mAb against chemotactic cytokines. mAb against IL-8 were found to have an anti-inflammatory effect on glycogen-induced peritonitis, IgG immune complex-induced alveolitis and dermal vasculitis.[127]

8. Aims of the study

The primary aim of this thesis was to investigate the role of human peritoneal mesothelial cells in the inflammatory response to bacterial invasion of the peritoneal cavity.

In an *in vitro* model system we studied the role of mesothelium derived chemoattractants during leukocyte migration across mesothelial cell monolayers. Another objective was to define the conditions of chemoattractant synthesis by cultured mesothelial cells and to investigate whether this inflammatory reaction correlates with bacterial peritonitis *in vivo*.

The leukocyte influx across activated mesothelium was studied with cultured human mesothelial-cell layers and isolated human leukocytes in a double chamber *in vitro* model system. The interactions between the specific adhesion receptors of both cell types and the possibility of modulation of this process was investigated. Furthermore, the transport of opsonins across cultured mesothelium was studied in the same model. The activation of peritoneal neutrophils during the onset of peritonitis was investigated in patients with CAPD-associated peritonitis.

The last part of this thesis describes the secretion by activated mesothelial cells of CA 125, a well-known tumor marker for ovarian carcinoma. Its secretion pattern and the circumstances under which cultured mesothelial cells secrete of CA 125 are described.

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2

POLARIZED SECRETION OF IL-8 BY HUMAN MESOTHELIAL CELLS: A ROLE IN NEUTROPHIL MIGRATION

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SUMMARY

We investigated the role of human mesothelium in an *in vitro* model of peritonitis with emphasis on the secretion of the neutrophil chemoattractant interleukin-8 and the migration of polymorphonuclear leukocytes (PMN) across monolayers of peritoneal mesothelial cells. PMN showed minimal migration across unactivated mesothelial monolayers (<2%). However, migration was induced after mesothelial cell activation by IL-1β (24%), and this induced migration was significantly blocked by antibodies against interleukin-8 (63% inhibition; $p \leq 0.01$). IL-1β-activated mesothelial monolayers were shown to secrete interleukin-8 in a polarized way, which was preferentially oriented towards the apical side of the monolayer. Our results indicate that the influx of PMN into the peritoneal cavity is, at least in part, controlled by the mesothelial cell layer of the peritoneal membrane.

INTRODUCTION

The abdominal cavity is lined with a monolayer of mesothelial cells resting on a basement membrane. The mesothelium provides a slippery, non-adhesive and non-thrombogenic surface that allows for smooth mobility of internal organs.[1] During peritonitis there is a massive influx of PMN from the circulation to the peritoneal fluid. These granulocytes have to migrate across the mesothelial lining to reach the peritoneal cavity. Recent *in vitro* experiments have shown that mesothelium may actively participate in the neutrophil influx through enhanced expression of the adhesion molecule ICAM-1 on its surface and by secretion of the neutrophil chemoattractant interleukin-8 (IL-8).[2] This investigation was carried out to examine further the functional role of the mesothelial lining in the migration of PMN. The pattern of IL-8 secretion and its influence on migration of PMN across mesothelial monolayers was studied in an *in vitro* model of peritonitis.

MATERIALS AND METHODS

Mesothelial cell culture

Mesothelial cells (MC) were isolated from human omentum, according to techniques modified from Nicholson et al.[3] and Wu et al.,[4] as described previously.[5] In brief, small pieces of omentum were removed early in the operative procedure from patients undergoing abdominal surgery for non-infectious conditions. All patients gave informed consent. The omentum was transferred to fluid containing 0.05% (w/v) trypsin-0.02% (w/v) EDTA (Gibco, Life Technologies, Paisley, UK). After 15 min the detached MC were pelleted by centrifugation at 1200 rpm for 5 min and resuspended in supplemented M-199 medium (Gibco). MC were grown until confluence in a 37°C, fully humidified, 5% CO_2 cabinet in polystyrene culture flasks (75 cm^2; Costar, Cambridge, MA) precoated with fibronectin. The identity of MC was demonstrated by the absence of von Willebrand factor staining[6] and the

presence of intracellular cytokeratins by using immunofluorescence with monoclonal antibodies[7] (Dakopatts, Glosstrup, Denmark).

IL-8 assay

MC were subcultured to confluent monolayers on polycarbonate membranes (0.4 µm pore size, 24.5 mm diam) of Transwell cell culture chamber inserts (Costar). The filters were precoated with fibronectin prior to addition of the MC. MC monolayers reached confluence in 5 days as determined by light-microscopy and by May-Grünwald/Giemsa staining. Inverted monolayers were cultured according to Parkos et al.[8] with minor modifications (Figure 1). For this purpose, a section of a 50 ml centrifuge tube (Costar) was gently, but tightly fixed to the inverted bottom part of each insert to prevent leakage of medium or cells. The inverted filters were also routinely treated with fibronectin. MC were added to the inverted inserts and allowed to attach overnight (Figure 1). Thereafter, the section of a 50 ml centrifuge tubes was removed, and the insert was placed upright into 6-well culture dishes. This culture was maintained for 5 days to reach MC confluency prior to use (Figure 1). Pretreatment of the monolayer with an optimal concentration of human recombinant IL-1β (rIL-1β; 25 U/ml; Genzyme Corporation, Cambridge, MA) did not influence the microscopic morphology of the confluent monolayers. All media were refreshed in the experiments before addition of rIL-1β to one of the compartments. At indicated intervals, samples from both compartments were taken and assayed for IL-8 by enzyme-linked immunosorbent assay (ELISA).[9] The results were expressed as absolute nanograms of IL-8 produced by the monolayer.

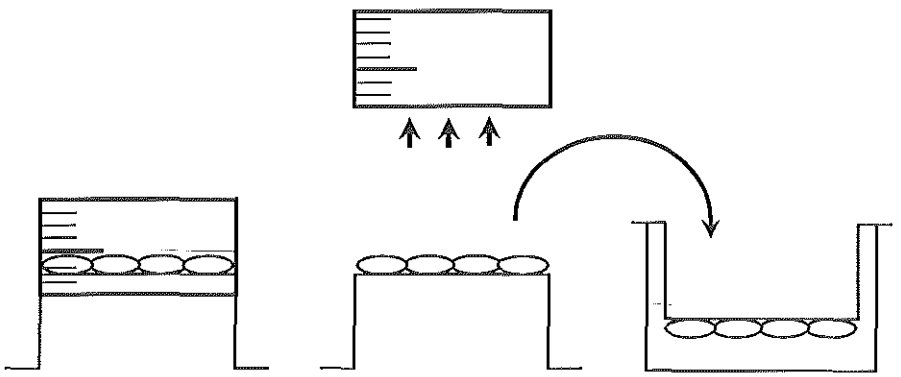

Figure 1. Preparation of an inverted mesothelial cell culture in a Transwell filter system. The insert containing the filter was inverted and a piece of a 50 ml centrifuge tube was tightly, but gently, fixed to the bottom part of the insert. The MC suspension were added to the inverted insert and allowed to attach overnight. The tube prevents leaking of the culture fluid from the inverted filter. The tube was then removed, the insert was placed upright in a 6-well culture dish and MC so attached to the underside of the filter were cultured for 5 additional days to reach a confluent mesothelial cell monolayer.

Transport of rIL-8 through MC monolayer

Recombinant IL-8 was obtained by transfecting *Escherichia coli* DH5 with the plasmid pMBL11 that contained cDNA encoding the 72-amino-acid species of human IL-8 (British Biotechnology Ltd., Oxford, UK).[9] Recombinant IL-8 was added to the apical side of non-activated MC monolayers, cultured on polycarbonate membranes (0.4 µm pore size, 24.5 mm diam). Over six hours serial samples from the lower compartments were taken and assayed for IL-8 by ELISA.[9]

Granulocyte isolation

Blood was obtained from healthy volunteers. Granulocytes were purified from a buffy coat by density gradient centrifugation over isotonic Percoll as described by Roos and De Boer.[10] Purity of the granulocytes was >98%, with >95% neutrophils. Viability measured by lactate dehydrogenase (LDH) release[11] was >95%.

Labeling of neutrophils

Freshly purified neutrophils were radiolabeled with ^{51}Cr according to Gallin *et al.*[12] Labeling of PMN with ^{51}Cr has no effect on chemotaxis of the neutrophil[12] and is therefore a standard method in adherence and migration experiments.[13] Briefly, neutrophils (10^7 cells/ml) suspended in 50% (v/v) M-199, 50% (v/v) RPMI-1640 (Gibco), supplemented with 0.1% (v/v) HSA, were incubated with 10 µCi of ^{51}Cr/ml (sodium chromate, 200 to 500 Ci/g; New England Nuclear, Boston, MA) at 37°C for 1 hour with gentle shaking. The cells were subsequently washed and resuspended in 50% (v/v) M-199, 50% (v/v) RPMI-1640, 0.5% (v/v) HSA (incubation medium). Viability after labeling remained >95%.

Migration assay

MC were subcultured on normal and inverted polycarbonate membranes (8.0 µm pore size, 24.5 mm diam; Costar) as already described. In the conventional configuration PMN were sedimented by gravity to the apical surface of the MC normally facing the abdominal cavity. Inverted filters were used to study migration in the more physiological direction (i.e. submesothelial tissue to abdominal fluid), thus permitting the PMN to approach and adhere to the basolateral side of the MC monolayers. In some experiments the monolayers were preincubated with an optimal concentration of rIL-1β (25 U/ml added to the lower compartment) for 6 hours prior to adding the PMN to the upper compartment. In all experiments the upper compartment of the cell culture chamber inserts was washed twice with incubation medium (50% (v/v) M-199, 50% (v/v) RPMI-1640, Gibco, supplemented with 0,5% (v/v) HSA), prewarmed to 37°C, before adding the PMN. However, the fluid in the lower compartment was not replaced, unless otherwise indicated. ^{51}Cr-labeled neutrophils (1×10^6 cells/ml), prewarmed to 37°C, were then added to the upper compartments. When indicated, mAb against IL-8 (mAb IL-8/6)[9] and in some experiments C5a (10^{-8} M, Sigma Diagnostics, St Louis, MO) or FMLP (10^{-6} M, Sigma) were added to the lower compartment 5 minutes prior to adding the PMN. The chambers were incubated in a 5% CO_2 incubator at 37°C for 30 minutes. After incubation, the fluid from both compartments was collected. The radioactivity present in the fluids as well as that on the filter, cut out of its cylindric container, were determined in a gammacounter. Recovery was always >92%. The radioactivity measured on the filter was taken to represent PMN adhesion. Light microscopic examination of the MC monolayers showed that most of the neutrophils were located at the margins of the MC. The extent of migration was calculated from the radioactivity found in the fluid sampled from the lower compartment. The results were expressed as percentages of the total radioactivity, i.e. PMN added to the chambers.

Measurement of chemotaxis

The Boyden chamber (48-well chemotaxis chamber, Neuro Probe Inc., Cabin John, MD) was used for measurement of PMN chemotaxis. The Boyden chamber contained a 8.0-µm migration filter on top of an 0.45-µm stop filter. Chemotactic stimulus, added to the lower compartment of the Boyden chamber, consisted of the supernatant of the basolateral or apical compartment of rIL-1β prestimulated mesothelial cell monolayers cultured on 0.4 µm filters, as described above. Recombinant IL-1β was added to the apical side of the monolayer. Culture medium and C5a (10^{-8} M, Sigma), diluted in culture medium, served as controls. When indicated, mAb against IL-8 (mAb IL-8/6)[9] was added to the apical supernatant 5 min prior to adding the PMN. PMN were suspended in medium containing 132 mM NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 6 mM KCl, 1.2 mM KH_2PO_4, 20 mM HEPES, 5.5 mM glucose, and 0.5% (w/v) HSA (CLB, Amsterdam, the Netherlands), pH 7.4, supplemented with heparin (10 IU/ml). 10^5 PMN were added to the upper compartment of the Boyden chamber and incubated for 1.5 h. Thereafter, the 8.0-µm Boyden chamber filters were stained, dehydrated with xylol and the number of

PMN was counted with a microscope at 400× magnification. Chemotaxis was defined as the mean of the number of PMN counted in five microscopic fields.

Transmesothelial electrical resistance measurement

The confluency and integrity of the mesothelial monolayer was tested by measuring the transmesothelial electrical resistance[14,15] (Millicel-ERS, Millipore Corporation, Bedford, MA) on both non-stimulated and prestimulated MC monolayers. MC were subcultured on normal and inverted polycarbonate membranes (8.0 µm pore size, 24.5 mm diam; Costar) as decribed above. The monolayers were cultured for 5 days to reach confluence. In some experiments the monolayers were preincubated with rIL-1β (25 U/ml, added to the apical side of the monolayers) for 6 hours as described earlier. In all experiments the medium of both compartments of the cell culture chamber inserts was replaced by medium M-199 without serum before measurement of the electrical resistance. Filters without MC monolayers served as controls. After the initial measurements the confluency of the MC monolayers was deliberately disrupted by replacing the medium with PBS containing EDTA (2 mM; Boehringer Mannheim GmbH, Mannheim, Germany). After incubation during 30 min in a 37°C, 5% CO_2 cabinet, the electrical resistance was again measured. Filters without MC monolayers served as controls.

RESULTS

Interleukin-8 secretion

The concentration of IL-8 was measured in the fluid of both compartments of the Transwell system. The mesothelial cell monolayer forms a barrier between these compartments, which enabled us to measure differences in apical and basolateral secretion. In the absence of deliberate stimulation, mesothelial cell monolayers secreted only low levels, i.e. less than 2 ng, of IL-8 (Figure 2). After the addition of rIL-1β to the apical surface of the monolayer, i.e. in the upper compartment, the mesothelial cells within 6 hours secreted large amounts of IL-8 in a polarized way: more IL-8 was found in the upper compartment than in the lower compartment of the system ($p \leq 0.05$, Figure 2). Surprisingly, when rIL-1β was introduced to the basolateral side of the monolayer, i.e. added to the lower compartment, IL-8 was also preferentially secreted into the upper compartment ($p \leq 0.05$, Figure 2). Mesothelial cells, thus, seem to create a gradient of IL-8 towards the apical side of the monolayer, irrespective of the direction of the inducing stimulus. To test this hypothesis further, inverted monolayers, in which the MC were adherent to the lower side of the filter, were likewise stimulated. Again, MC IL-8 secretion was found to be polarized towards the apical side of the monolayer ($p \leq 0.05$, Figure 2).

The transport of exogenous rIL-8 was tested through non-activated mesothelial monolayers, cultured on normal filters (0.4 µm pore size) to study the potential confounding influence of active or passive back-diffusion of secreted IL-8 through the monolayers. Within six hours part of the rIL-8 added to the upper compartment was detected in the lower compartment, indicating that active or passive transport of IL-8 through the monolayer did occur (Figure 3).

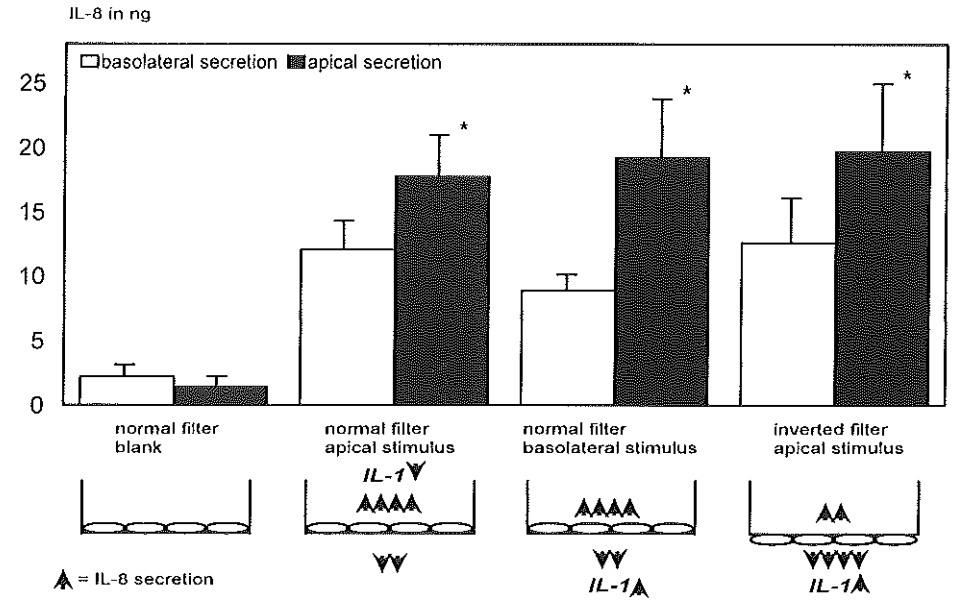

Figure 2. Secretion of IL-8 by confluent monolayers of MC. Normal and inverted 0.4 µm filters were used. MC were stimulated by addition of rIL-1β (25 U/ml) to the apical or basolateral side of the monolayer, and the IL-8 secretion was measured after 6 hours. Results are expressed as the mean ± SEM in nanograms of eight separate experiments. *Apical concentration significantly higher than the basolateral concentration of IL-8 ($p \leq 0.05$, two tailed paired Student *t*-test).

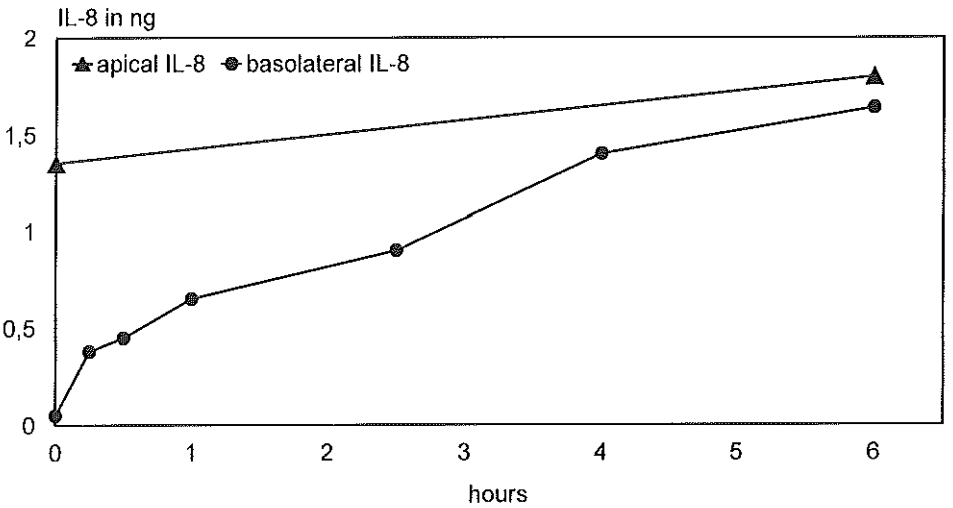

Figure 3. Representative figure of the time-course of rIL-8 transport through a non-stimulated MC monolayer. A normal 0.4 µm filter was used, rIL-8 was added to the apical side of the monolayer at 0 hours. At various times samples of the lower compartment were taken and measured in an IL-8 ELISA.

Diffusion of rIL-8 through a filter (0.4 µm, 24.5 mm diam) without a MC monolayer was tested to control for the possible influence of the design of the Transwell system. The Transwell filter itself formed no major barrier to rIL-8 diffusion, nor was coating of the filter with fibronectin of influence (data not shown).

Migration

PMN adhesion and migration across non-activated mesothelial monolayers in normal and inverted filters was minimal (<2% in 30 min; Table 1). However, migration of PMN across the monolayer was invariably found after MC stimulation with rIL-1β (Table 1). Inversion of the filter resulted in even higher levels of PMN migration (96% increase, $p \leq 0.01$, table 1), which correlated well with the difference in amounts of IL-8 found in the lower compartments, between normal and inverted filters (Figure 1).

The presumed difference in barrier between normal and inverted filters for neutrophils to reach the lower compartment was determined by using C5a or FMLP as chemoattractant in otherwise unstimulated MC monolayers in PMN adherence to and migration across the filters. There was no difference in neutrophil adherence to and migration across normal or inverted mesothelial cell monolayers induced by these chemoattractants, indicating no difference in barrier for neutrophils between the two different ways of culturing a confluent MC monolayer on the filters (data not shown).

Increase in adherence to the MC monolayer, but not a significant increase in migration across activated inverted filters was seen when the medium in the lower compartment was replaced with fresh medium before adding the PMN (Table 1). However, when the medium in the lower compartment was left *in situ*, significant increase in migration was observed ($p \leq 0.01$, Table 1).

To delineate the role of MC-excreted IL-8 further, the effect of a mAb against IL-8 (mAb IL-8/6) was tested in the set-up with stimulated inverted filters. Migration of PMN across a monolayer of MC was reduced by 63% ($p \leq 0.01$), indicating that IL-8 was the major chemotactic moiety excreted by MC (Table 1).

Addition of the chemoattractant C5a to the lower compartment resulted in extensive adherence and high levels of migration of PMN in otherwise unstimulated inverted filters (Table 1).

mAb IL-8/6 did not influence the adherence to and migration of PMN across non-activated mesothelial monolayers (data not shown). Furthermore, the adherence and migration of PMN induced by C5a was also not affected by mAb IL-8/6 (data not shown).

Table 1. PMN adherence and migration across MC monolayers

	% adhesion	% migration
Normal filters		
Blank	3.0 ± 0.5	1.7 ± 1.1
Apical stimulus	10.9 ± 2.9	12.1 ± 2.7
Inverted filters		
Blank	3.2 ± 0.5	1.8 ± 1.5
Apical stimulus	8.6 ± 1.2	23.7 ± 2.3**[1,2]
Apical stimulus #	20.8 ± 5.4	3.2 ± 1.4
Apical stimulus, mAb IL-8	15.2 ± 2.5	8.8 ± 2.2**[3]
C5a	19.0 ± 4.5	30.6 ± 7.8*[4]

Table 1. Mesothelial cells were cultured on 8.0-µm filters. Neutrophil adherence to and migration across normal and inverted monolayers of MC were measured 30 min after adding the PMN. Results are expressed as the mean ± SEM of four to six experiments performed on separate occasions. # Lower compartment was washed twice with incubation medium before adding the PMN. mAb IL-8/6 (1µg/ml) was added to the lower compartment 5 min prior to adding the PMN. In some experiments neutrophils were attracted to the lower compartment by C5a (10^{-8} M). **[1] Migration across an inverted filter was significantly higher compared to migration across a normal filter ($p \leq 0.01$). **[2] Significant increase in migration after stimulation of the MC with rIL-1β, compared to the migration across non-stimulated MC monolayers ($p \leq 0.01$); **[3] mAb against IL-8 significantly inhibited migration across stimulated MC monolayers ($p \leq 0.01$); *[4] C5a induced a significant increase in migration across non-stimulated MC monolayers ($p \leq 0.05$). All statistic analyses were performed using the two-tailed unpaired Student's *t*-test.

Chemotaxis of mesothelial supernatant

The difference in PMN chemotaxis between basolateral and apical supernatant, obtained from rIL-1β prestimulated MC monolayers cultured on 0.4 µm filters was shown in the Boyden chamber (Table 2). Supernatant obtained from the basolateral compartment of prestimulated MC monolayers induced significantly less chemotaxis compared to supernatant from the apical compartment ($p \leq 0.05$, Table 2). It was again shown in the Boyden chamber that IL-8 was the major chemoattractant secreted by stimulated MC monolayers, as mAb IL-8 added to apical supernatant reduced the chemotaxis of PMN with 74.8% ($p \leq 0.01$, Table 2).

Transmesothelial electrical resistance

To establish the confluency and integrity of the MC monolayers used in our experiments, the transmesothelial electric resistance of typical monolayers were repeatedly measured. Mesothelial monolayers after 5 days of culture were shown to give a significant increase in electrical resistance ($p \leq 0.01$), compared to bare filters without an MC monolayer (Table 3). Neither culture of the MC on inverted filters nor prestimulation of the MC with an optimal concentration of IL-1β altered the electrical resistance (Table 3).

Table 2. Boyden chamber measurement of PMN chemotaxis

Chemotactic stimulus	number of PMN migrated			
Culture medium	12.8	±	2.2	
Basolateral supernatant	58.0	±	9.2	
Apical supernatant	90.5	±	0.9	*[1]
Apical supernatant, mAb IL-8	22.8	±	7.6	**[2]
C5a	180.8	±	45.0	

Table 2. Supernatant was obtained from the basolateral or apical compartment of rIL-β prestimulated mesothelial cell monolayers, cultured on 0.4 μm filters. rIL-1β was added to the apical side of the monolayer. Culture medium and C5a diluted in culture medium served as negative and positive controls, respectively. Results are expressed as the mean ± S.E.M. of four experiments performed on separate occasions. All stimuli gave a significant increase in PMN chemotaxis compared to incubation medium ($p \leq 0.01$). *[1]Number of PMN significantly higher compared to basolateral supernatant ($p \leq 0.05$). **[2]Significant inhibition of mAb against IL-8 on PMN chemotaxis added to apical supernatant ($p \leq 0.01$). All statistic analyses were performed with the two-tailed paired Student's *t*-test.

After 30 min incubation with PBS/EDTA, the electrical resistance of all filters had fallen to the same level as the control filter without an MC monolayer (data not shown).

Table 3. Measurement of transmesothelial electrical resistance

	normal filter	inverted filter
Filter without MC monolayer	104.6 ± 2.5	104.6 ± 2.5
Filter with MC monolayer		
No stimulus	149.8 ± 3.6 **	157.0 ± 2.7 **
Apical stimulus of IL-1β	153.0 ± 8.1 **	154.6 ± 6.8 **

Table 3. Mesothelial cells were cultured on 8.0 μm filters. Transmesothelial electrical resistance was measured 6 hours after adding rIL-1β to the apical side of the monolayer. Results are expressed in Ohms as the mean ± SEM of five separate experiments. **Electrical resistance was significantly higher compared to a control filter without a MC monolayer ($p \leq 0.01$, two tailed paired Student's *t*-test).

DISCUSSION

The entry of neutrophils into tissues or body cavities is a prerequisite for an early and adequate inactivation of invading micro-organisms. Results of previous studies indicate that the presence of a gradient of chemotactic mediators generated at the site of inflammation, e.g. formylmethionyl peptides of bacterial origin, the anaphylatoxin C5a, lipid-derived mediators such as platelet-activating factor (PAF), or IL-8, is of utmost importance for transendothelial migration of PMN.[13,16] Our results indicate that in the peritoneal

cavity the mesothelium itself, once activated, is able to create a functional chemotactic gradient of IL-8 in the physiologically relevant direction.
The apical surface of peritoneal mesothelium facing the abdominal cavity is profusely carpeted with microvilli.[17] This extension of apical surface compared to the basolateral surface of mesothelium might cause the difference in apical versus basolateral secretion of IL-8 to be created. Furthermore, mesothelium is known to possess sophisticated junctional complexes that might allow a gradient of IL-8 to be maintained.[17] Measurements of the transmesothelial electrical resistance proved indeed that confluent MC monolayers create a tightly joined layer of cells in which the polarized secretion of cytokines and transmesothelial migration of PMN can be studied. However, in spite of its polarized secretion we also found some passive or active transport of IL-8 across non-stimulated MC monolayers. This may be because part of the apically secreted IL-8 is secondarily transported or passively diffused to the basolateral side of the monolayer. The small molecular size of the cytokine IL-8 (8 kD) probably facilitates the diffusion through the MC monolayer. It is known from patients on chronic peritoneal dialysis and from studies in experimental animals that the peritoneal clearance of molecules decreases as the molecular size of the inoculum increases.[18,19] This concurs with the results from a previous study[5] in which a greater proportional difference between preferential apical against basolateral secretion was found by activated MC monolayers for the tumor marker CA 125 (220 kD).
Microscopic E.M. analysis of mesothelial cells has revealed an abundance of rough endoplasmic reticulum, well-developed Golgi complexes and large nuclei showing predominance of euchromatin over heterochromatin,[17] indicating that mesothelium is capable of protein synthesis and secretory activity. Recently, activated mesothelial cells were shown to produce several cytokines, including granulocyte colony-stimulating factor (CSF), monocyte chemotactic protein-1 (MCP-1), interleukin-6 (IL-6),[20] IL-8[21-23] and transforming growth factor-β (TGF-β).[24] We now report that MC are capable to secrete at least one such cytokine, IL-8, in a polarized fashion, which allows it to be functionally active and physiologically relevant. Indeed, high levels of IL-8 can be found in dialysate samples from patients on peritoneal dialysis during episodes of bacterial peritonitis.[25,26] The functional activity of the mesothelial IL-8 gradient was shown in our study by different PMN migration experiments. Apical derived supernatant from stimulated MC monolayers induced higher chemotactic activity compared to basolateral supernatant. Even more important than the absolute difference in chemotactic potency between the two sides of the cell-layer is probably the presence of the gradient itself. Neutrophil movements are fueled by the presence of a chemotactic gradient of mediators, e.g. IL-8, generated at the site of inflammation.[16] The pattern of polarized secretion of IL-

8 as we found, which is apically directed irrespective of the site of the inducing stimulus, is not unique. Recently, the same polarized type of secretion was found for CA 125 by MC[5] and for the cytokine MCP-1 by rat type 2 alveolar epithelial cells.[27] IL-8 was also found to be secreted in a polarized fashion by other cell types. McCormick *et al.*[28] studied the IL-8 secretion of activated T84 epithelial cells in the Transwell system and found that IL-8 was preferentially secreted to the basolateral side of the monolayer, indicating that the Transwell filter forms no barrier for IL-8 diffusion.

The secretion of other products by MC, or of cytokines by other human cell types, may likewise be secreted in a polarized fashion, such as we described for IL-8 by MC. This would concur with the physiological cascade of inflammatory events as observed in nature.

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3

NEUTROPHIL ADHERENCE TO AND MIGRATION ACROSS MONOLAYERS OF HUMAN PERITONEAL MESOTHELIAL CELLS

The role of mesothelium in the influx of neutrophils during peritonitis

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ABSTRACT

Increased adherence to and subsequent migration of leukocytes across cultured human peritoneal mesothelial cell monolayers takes place after pretreatment of the mesothelial cells with IL-1β. The contribution of the leukocyte β_2 integrins (CD11/CD18), the mesothelial adhesion protein ICAM-1 and the role of the cytokines IL-8, PAF and TGF-β were studied in a three-dimensional model system for neutrophil-mesothelial monolayer interaction. PMN showed minimal adherence to and migration across unactivated mesothelial monolayers, despite an extensive amount of ICAM-1 on the mesothelial membrane. Pretreatment of the monolayers with rIL-1β induced enhanced PMN adherence to the mesothelial monolayer together with a further increase in ICAM-1 expression on the mesothelial membrane. PMN migration was observed across rIL-1β activated MC monolayers whenever secreted cytokines were present during migration. mAb R6.5 against ICAM-1 and mAb CLB-LFA1/1 against CD18 both reduced the migration of PMN across mesothelial monolayers with a predominant inhibitory effect of CLB-LFA1/1, indicating a significant role of the β_2 integrins of PMN in this process. IL-8 was the major cytokine synthesized by the mesothelial cells to stimulate the migration of PMN; both PAF and TGF-β had a more modest role in our system. Adherence of PMN to mesothelial cell monolayers was not dependent on these latter cytokines. Neuraminidase did not have any effect, indicating that selectins were not involved in the adherence process. rIL-1β pretreated mesothelial cells induced a rapid increase in intracellular Ca^{2+} in PMN; Actinomycin D blocked this effect and was also able to prevent adhesion of neutrophils to activated mesothelial cell monolayers. Neutrophil migration across activated cultured mesothelial cells is a cascade of events in which the mesothelial cells are actively involved.

INTRODUCTION

The neutrophilic granulocytes are the initial cells found at areas of tissue inflammation.[1, 2] It is known from previous studies that the neutrophil influx is a well-coordinated process, in which adherence of neutrophils to endothelial cells and subsequent migration across the endothelial barrier into the adjacent tissue takes place through specific adhesion receptors on both types of cells and guidance by locally produced chemotactic factors.[3-5] In case of peritonitis, the neutrophils not only have to migrate across an endothelial cell layer when leaving the circulation but also across a monolayer of mesothelial cells before they can enter the peritoneal cavity. Mesothelium lines the abdominal cavity and provides a slippery, non-

adhesive and non-thrombogenic surface that allows for smooth mobility of internal organs.[6] In vitro experiments have shown that activated mesothelium increases the expression of adhesion protein ICAM-1 on its membranes[7] and actively secretes IL-8[8, 9] which is chemotactic for the neutrophils.[10] However, little is currently known about the process of transmesothelial cell migration of PMN. Therefore, we studied in a three-dimensional model both the adhesion receptors and the cytokines involved in neutrophil adherence to and migration across mesothelial cell monolayers. It was of special interest to establish whether the mesothelium itself was actively involved in this part of the inflammatory response, as observed during bacterial peritonitis.

MATERIALS AND METHODS

Mesothelial cell culture

Mesothelial cells (MC) were isolated from human omentum, as described before.[11] The identity of MC was demonstrated by the absence of von Willebrand factor staining[12] and the presence of intracellular cytokeratins[13] by immunofluorescence with monoclonal antibodies (Dakopatts, Glosstrup, Denmark).

Granulocyte isolation

Blood was obtained from healthy volunteers. Granulocytes were purified from a buffy coat by density gradient centrifugation over isotonic Percoll as described by Roos and De Boer.[14] Purity of the granulocytes was ≥98%, with ≥95% neutrophils. Viability measured by lactate dehydrogenase (LDH) release[15] was ≥95%.

Labeling of the neutrophils

Freshly purified neutrophils were radiolabeled with ^{51}Cr according to Gallin et al.[16] Briefly, neutrophils (10^7 cells/ml) were incubated with 1 µCi of ^{51}Cr per 10^5 cells (sodium chromate, 200 to 500 Ci/g; New England Nuclear, Boston, MA) in 50% (v/v) M-199 (Gibco, Life Technologies, Gaithersburg, MD), 50% (v/v) RPMI-1640 (Gibco), supplemented with 0.1% (v/v) HSA, at 37°C for 45 min with gentle shaking. Subsequently, the cells were washed and resuspended in 50% (v/v) M-199, 50% (v/v) RPMI-1640, 0.5% (v/v) HSA (incubation medium). Viability after labeling remained ≥95%.

Adhesion and migration assay

Both PMN adhesion to and migration of PMN across MC monolayers were studied on inverted mesothelial monolayers, in the physiologically relevant direction (submesothelial tissue to abdominal fluid), thus permitting the PMN to approach and adhere to the basolateral side of the monolayer. MC were subcultured to confluent inverted monolayers on polycarbonate membranes (8.0 µm pore size, 24.5 mm diam) of Transwell cell culture chamber inserts (Costar, Cambridge, MA), as described.[9] MC monolayers reached confluency in five days as determined by phase-contrast microscopy, by microscopy of May-Grünwald/Giemsa stained filters and by electrical resistance measurements.[9] In some experiments the monolayers were preincubated with an optimal concentration of 25 U/ml of rIL-1β (Genzyme, Corporation, Cambridge, MA), added to the lower compartment 6 hours prior to adding the PMN. rIL-1β had no effect on the morphology of the cells, nor did it influence the electrical resistance of the monolayers.[9] In all experiments the upper compartment of the cell culture chamber inserts was washed twice with incubation medium, prewarmed to 37°C, just prior to adding the PMN. However, the fluid in the lower compartment was not

replaced unless otherwise stated. ^{51}Cr-labeled neutrophils (10^6 cells/ml) prewarmed to 37°C were subsequently added to the upper compartments. When indicated, MC and neutrophils were both incubated with the following mAbs, during 15 min before the neutrophils were added: mAb CLB-LFA1/1 (IgG_1), which recognizes the common CR3 β chain CD18,[17] mAb R6.5 (IgG_{2a}) against ICAM-1,[18] (a kind gift of Dr. R. Rothlein, Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, CT) and mAb IL-8/6 against human IL-8 (a kind gift of Dr. L.A. Aarden, CLB, Amsterdam, the Netherlands). mAb against TGF-β was obtained from R&D systems, Minneapolis, MN. The mAbs were added in concentrations which showed optimal inhibitory effects and remained present during the assay. The PAF receptor antagonist WEB 2086[19] (10 μM; a kind gift of Dr. H. Heuer, Boehringer Mannheim GmbH, Mannheim, Germany), was likewise used. The stock of WEB 2086 was diluted in DMSO; the same amount of DMSO was added to control experiments without effect on adherence to and migration of PMN across MC monolayers. The chamber plates were incubated at 37°C in a 5% CO_2 incubator for 30 min. After incubation, the fluid of each compartment was collected. The amount of radioactivity present in the fluids as well that of the filter, cut out of its cylindric container, was determined in a gamma counter. Recovery of radiolabel was always ≥92%. The radioactivity measured in the filter was taken to represent PMN adhesion. The extent of migration was calculated from the radioactivity found in the fluid of the lower compartment. The results were expressed as percentages of the total radioactivity, i.e. PMN, added to the chambers.

Determination of surface antigen expression

The expression of the adhesion molecules ELAM-1 and ICAM-1 by mesothelial cells was determined by FACS analysis. MC were subcultured to confluent monolayers in 6-well culture dishes (Costar, Cambridge, MA). Activation of the MC took place by adding IL-1β (25 U/ml) to the MC-monolayers. When indicated, actinomycin D (1μg/ml; Sigma Co., St Louis, MO) was added 15 min before activation of the MC with rIL-1β. As control served a well that was not incubated with either actinomycin D or IL-1β. After 6 h, the MC were non-enzymatically detached by adding PBS/EDTA (2 mM) during 15 min at 37°C. MC in suspension were washed with ice-cold PBS and subsequently incubated with mAb against ELAM-1 (ENA 2)[20] or ICAM-1 (RR1/1 [IgG_1])[21] for 30 min at 4°C. Thereafter, the cells were washed twice in excess of ice-cold PBS, and the procedure was repeated with FITC-labeled goat-anti-mouse-Ig for another 30 min at 4°C. After two washes, mAb binding was quantified for 10,000 cells with a FACScan (Becton Dickinson, Mountain View, CA) and was expressed as Mean Fluorescence Intensity (MFI). The inhibitory effect of actinomycin D on mesothelial monolayers was checked by measurement of the IL-8 secretion, which is known to be dependent on de novo protein synthesis.[8] The IL-8 secretion was measured by ELISA, according to Hack et al.[22]

Adhesion assay

MC were subcultured to confluent monolayers in 6-well culture dishes (Costar). IL-1β (25 U/ml) was added 6 hours before addition of the PMN; as control served a well that was not incubated with IL-1β. When indicated, actinomycin D was added as well and its inhibitory effect checked as described earlier. Phorbol myristate acetate (PMA, 100 ng/ml; Sigma Co.) was used for prestimulation of the neutrophils before addition to the MC monolayers. Treatment with neuraminidase (type V, from *Clostridium perfringens*; Sigma) was used to remove terminal sialic acid residues,[23] the counter structures for selectins. Neuraminidase was added to the PMN and MC monolayers in a concentration of 0.2 U/ml 1 h prior to addition of the neutrophils to the monolayers. Neuraminidase treatment of the MC monolayers caused no morphologically visible toxic effect, which concurs with previous findings of Render et al. with neuraminidase treatment of endothelial cells.[24] mAb CSLEX-1 (IgM)[25] against the sialylated Lewis-x antigen (SLe^x) and mAb CLB-B4.3 (IgM)[26] against the Lewis-x antigen (Le^x) were used for measuring, by FACS analysis, the effect of neuraminidase on the change of SLe^x into Le^x on the cell membrane of neutrophils. In all

experiments the wells were washed twice with incubation medium before ^{51}Cr-labeled neutrophils (10^6 cells) prewarmed to 37°C were added to the wells. The plates were incubated at 37°C in a 5% CO_2 incubator for 30 min. After incubation, the fluid of each well was collected and the wells were very gently washed once to remove the non-adherent PMN. Thereafter, the MC were detached from the wells by adding Triton-X-100 1% (v/v) to the wells, during 30 min in the CO_2 incubator. The amount of radioactivity present in the extracellular fluid as well as that of the detached MC, was determined in a gammacounter. Recovery was always ≥92%. The radioactivity measured in the detached MC was taken to represent PMN adhesion. The results were expressed as percentage of the total radioactivity, i.e. PMN added to the wells.

Measurement of cytosolic free Ca^{2+} during adherence of PMN to mesothelial cells

Cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) was measured as previously described.[27,28] Briefly, neutrophils prewarmed for 5 min at 37°C, at a concentration of 2×10^7 cells/ml in medium for cell suspensions (see above) were incubated with 0.5 µM indo-1/AM (Molecular Probes) for 40 min at 37°C. After two washes, the cells were resuspended to 2×10^6 cells/ml in medium for cell suspensions and kept at room temperature. Fluorescence intensity was determined in a spectrofluorometer (model RF-540; Shimadzu Corporation, Kyoto, Japan) at 37°C under continuous stirring. Excitation and emission wavelengths were 340 and 390 nm, respectively. Calibration of indo-1 fluorescence was achieved by saturation of trapped indo-1 with Ca^{2+} after permeabilization of the cells with digitonin (5 µM), followed by quenching with Mn^{2+} (0.5 mM). A Kd of 250 nM for the Indo-1 Ca^{2+} complex was used for the calculation of $[Ca^{2+}]_i$.[29] Measurement of $[Ca^{2+}]_i$ in neutrophils was studied during adherence to non-stimulated or IL-1β prestimulated mesothelial cells, in the presence or absence of actinomycin D. Confluent MC monolayers were incubated with actinomycin D and IL-1β as described earlier. Subsequently, the monolayers were washed once with PBS and nonenzymatically detached (1.5 mM EDTA, 20-30 min). The cells were washed twice and brought to 7×10^6 cells/ml in medium for cell suspensions (final MC to neutrophil ratio of 1:3). The role of the PMN β_2 integrins and the different mesothelial-derived cytokines in the Ca^{2+} homeostasis of neutrophils adherence to IL-1β prestimulated MC was tested by incubation of both MC and PMN with mAb CLB-LFA1/1, mAb anti-TGF-β, mAb anti-IL-8, or WEB 2086 15 min prior to adding the PMN to the MC.

RESULTS

Neutrophil adherence and migration

"Spontaneous" adherence and migration of PMN across non-activated mesothelial monolayers was minimal; essentially less than 5% of the cells adhered or migrated in this setting (Figure 1). Testing of IL-1β-activated mesothelial monolayers without the influence of secreted cytokines was performed by replacing both compartments of the transwell system with fresh medium just prior to adding PMN to the upper compartment. It resulted in significant adherence of PMN to the mesothelial monolayers (21%) but had little effect on the neutrophil migration (3%) (Figure 1). Incubation of the PMN and mesothelial cells with mAbs against IL-8 or TGF-β, and incubation with the PAF-receptor antagonist WEB 2086 did not interfere with the observed adherence (Table 1, *upper part*). In contrast, significant adherence (7%) as well as migration (23%) were found when MC were activated and the lower compartment was not replaced during the assay (Figure 1). When this assay was repeated in the presence of mAb R6.5 (which recognizes

ICAM-1) or mAb CLB-LFA1/1 (specific for CD18) a significant reduction of migration of the PMN (27% and 87% inhibition, respectively) was observed (Figure 1). The influence of the cytokines PAF, TGF-β and IL-8 produced by activated mesothelial cells was tested by addition of WEB 2086, mAb TGF-β or mAb IL-8/6, which resulted in 29%, 35% and 65% inhibition of migration, respectively (Table 1, *lower part*). The combined use of WEB 2086, mAb TGF-β and mAb IL-8/6 did not result in an synergistic inhibitory effect on PMN migration (data not shown).

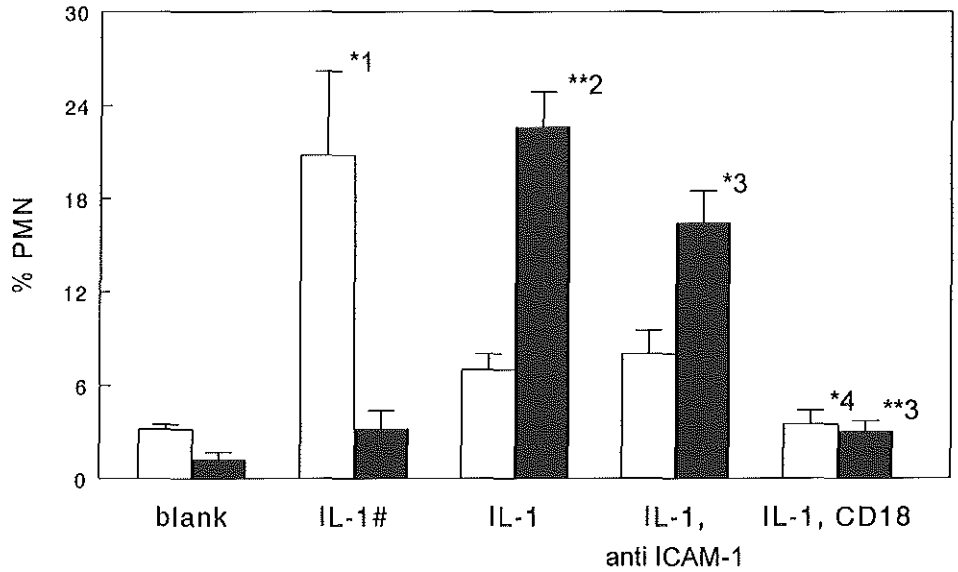

Figure 1
Involvement of ICAM-1 and CD18 proteins in the interaction of neutrophils with IL-1β-treated MC monolayers. Mesothelial cells were cultured on inverted filters with 8.0-μm pores. Neutrophil adherence (open bars) to and migration (closed bars) across inverted monolayers of MC were measured 30 min after adding the PMN. Blank represents adherence and migration across resting MC; IL-1# represents filters with IL-1β-activated MC, but the lower compartment was replaced with fresh medium before adding the PMN. Results are expressed as the mean ± SEM of three to five experiments performed on separate occasions. [1]PMN adherence significantly higher compared to resting MC. [2]Migration significantly higher compared to resting MC. [3]Significant inhibition of PMN migration compared to IL-1β-stimulated MC monolayers without CD18 or ICAM-1 antibodies. [4]Significant inhibition of PMN adherence compared to IL-1β-stimulated MC monolayers without CD18 mAb. (* $p \leq 0.05$, ** $p \leq 0.01$; unpaired *t*-test.)

Adhesion receptors

Cultured resting mesothelial cells, isolated from human omentum, already possessed a significant copy number of the adhesion receptor ICAM-1 on their membranes (Figure 2A). However, we found that adherence of PMN to such resting MC monolayers was minimal (Figure 1 and 2B).

Table 1. PMN adherence and migration across IL-1β-activated inverted monolayers of MC

chemotactic inhibitor	% adhesion	% migration	
Without secreted cytokines in the lower compartment			
None	24.3 ± 2.3	1.9 ± 0.5	
mAb TGF-β	23.7 ± 1.1	3.9 ± 0.2	
WEB 2086	26.4 ± 2.1	4.7 ± 0.5	
mAb IL-8	24.3 ± 3.4	2.0 ± 0.3	
With secreted cytokines in the lower compartment			
None	7.0 ± 1.0	22.6 ± 2.3	
mAb TGF-β	7.4 ± 2.0	14.6 ± 2.3	**[1]
WEB 2086	7.2 ± 1.4	16.0 ± 1.1	*[1]
mAb IL-8	13.6 ± 2.4	7.8 ± 1.4	*[1]

Table 1. IL-1β-activated MC monolayers were incubated with 51Cr-labeled PMN in the presence or absence of indicated chemotactic inhibitors. Adherence and migration of PMN were determined after 30 min.The results are expressed as the mean ± SEM of three to five experiments performed on separate occasions (* $p \leq 0.05$; ** $p \leq 0.01$, unpaired *t*-test). [1] Significant inhibition of migration of PMN across MC monolayers.

Treatment of MC with IL-1β somewhat enhanced their ICAM-1 surface expression (Figure 2A) but much more significantly, increased the adherence of PMN (Figure 1 and 2B). IL-1β incubation of MC in the presence of actinomycin D still induced an increase in ICAM-1 expression on the MC surface (Figure 2A) but did not lead to an increase in neutrophil adherence (Figure 2B). ELAM-1 expression was below the detection limit on the membrane of either resting or activated MC (data not shown).

Neutrophil adherence

Prior incubation of PMN with PMA resulted in a profound increase in their ability to adhere to mesothelial cell monolayers in culture dishes (Figure 3). The PMA-induced adherence of PMN to otherwise unstimulated MC monolayers turned out to be a CD11/CD18-mediated event as well, since the CD18-specific mAb CLB-LFA1/1 inhibited PMA-induced adherence by 86.3% (Figure 3). In contrast, preincubation of the neutrophils with either neuraminidase or actinomycin D did not significantly influence the PMA-induced adherence. Neuraminidase treatment of PMN and mesothelial cells did not affect PMN adherence to either resting or IL-1β-activated mesothelial monolayers, and also, as mentioned above, did not affect adherence of PMA-stimulated PMN to resting MC (Figure 3).

Figure 2A

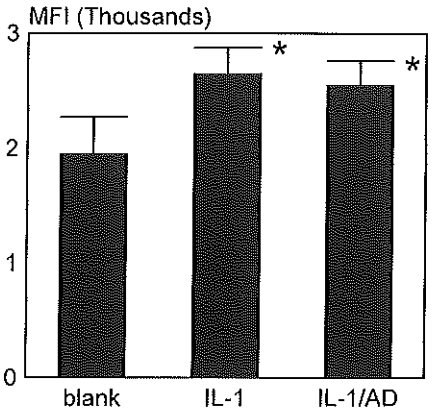

Figure 2B

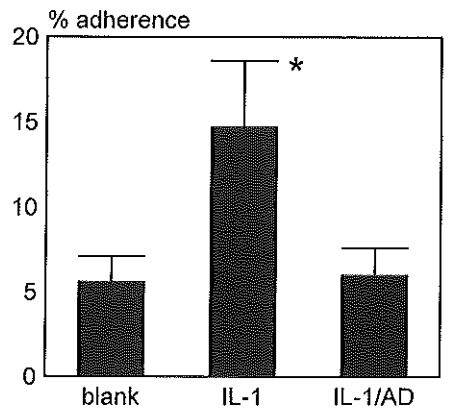

Figure 2. Requirement of protein synthesis by IL-1β-treated MC for surface expression of ICAM-1 (Fig 2A) and binding of neutrophils (Fig 2B). Mesothelial cells were cultured on 6-well culture dishes.

Figure 2A- ICAM-1 expression was measured on resting and IL-1β-prestimulated MC. The results are expressed in ICAM-1-specific MFI ± SEM for five experiments performed on separate occasions. * ICAM-1 expression significantly higher compared to ICAM-1 expression on resting MC. (* $p \leq 0.05$; paired *t*-test.)

Figure 2B- Adherence of PMN to resting and IL-1β-prestimulated MC 30 min after the addition of the PMN. The results are expressed as the mean percentage of adherent PMN ± SEM based on five experiments performed on separate occasions. * Adherence significantly higher compared to adherence of PMN to resting MC or actinomycin D pre-treated activated MC. (* $p \leq 0.05$; paired *t*-test.)

The lack of adherence of PMN to MC monolayers prestimulated with IL-1β in the presence of actinomycin D could in part be amended by, simultaneously with the PMN, adding a supernatant from MC monolayers that had been prestimulated with IL-1β in the absence of actinomycin D (Figure 3).

Measurement of cytosolic free Ca^{2+} during adherence of PMN to mesothelial cells

IL-1β prestimulated MC induced a rapid change in $[Ca^{2+}]_i$ in the PMN (Table 2). This Ca^{2+} flux was not influenced by mAb against IL-8 or TGF-β, or by WEB 2086 (data not shown), nor was it changed by mAb CLB-LFA1/1 against CD18 (data not shown). $F(ab')_2$-fragments of the CD18 IgG antibodies had the same lack of effect as intact mAb. An increase in Ca^{2+} flux was not found after adding non-activated MC, or MC prestimulated with IL-1β in the presence of actinomycin D to the PMN (Table 2). In reverse, incubation of PMN with indo-1 loaded MC did not show these rapid $[Ca^{2+}]_i$ changes (data not shown). Activation of the MC with IL-1β or incubation with actinomycin D had no effect in this set-up.

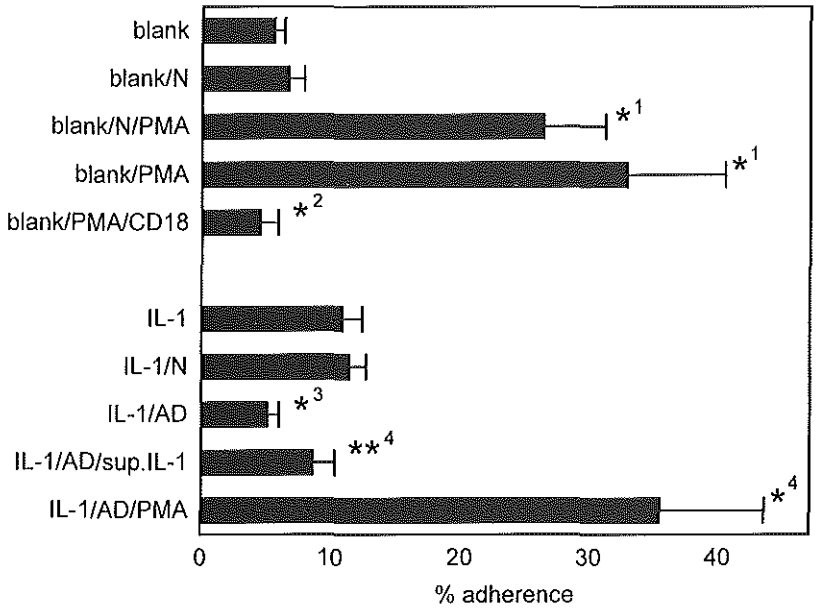

Figure 3. The effect of neuraminidase (N) and PMA on neutrophil adherence to resting and IL-1β-prestimulated MC monolayers. Results are expressed as mean ± SEM % PMN adhered based on experiments performed on separate occasions (* $p \leq 0.05$; ** $p \leq 0.01$, paired *t*-test). [1] Increase in adherence of PMN by PMA compared to adherence without additions (blank). [2] Inhibitory effect of PMN adherence by CD18 Ab compared to PMA-stimulated neutrophil adherence. [3] Inhibitory effect of PMN adherence compared to IL-1β-induced neutrophil adherence. [4] Increased adherence of PMN compared to neutrophil adherence to IL-1β-induced, actinomycin D (AD)-treated MC monolayers. Sup. IL-1 denotes supernatant from IL-1β-stimulated MC.

Table 2. Cytosolic rise of calcium ions in neutrophils after incubation with resting and IL-1β activated MC.

PMN incubation with	$[Ca^{2+}]_i$ (nM)
Resting MC	141 ± 45
IL-1β activated MC	509 ± 139 *1
IL-1β/AD activated MC	76 ± 10
FMLP (control)	1737 ± 122 **2

Table 2. PMN were suspended with indicated MC preparations after which PMN cytosolic Ca^{2+} was determined. The results are expressed as the mean ± SEM peak $[Ca^{2+}]_i$ of three experiments performed on separate occasions. *$p \leq 0.05$; ** $p \leq 0.01$, paired *t*-test. [1] Significant increase in $[Ca^{2+}]_i$ in PMN compared to non-activated MC or IL-1β/AD treated mesothelial cells. [2] Significant increase in $[Ca^{2+}]_i$ in PMN compared to non-activated, IL-1β, or IL-1β/AD treated MC.

DISCUSSION

During peritonitis, neutrophils have to migrate across a mesothelial cell layer before entering the peritoneal cavity. From the data generated in this study we conclude that neutrophil adherence and subsequent migration across cultured mesothelium can be seen as a cascade of events controlled by different regulating mechanisms, all originating from activated mesothelium itself.

First, mesothelium is known to synthesize various cytokines[8, 9, 30, 31] that are involved in neutrophil chemotaxis.[10, 32-34] Our results provide insight into the qualitative roles of these mesothelial-derived cytokines in neutrophil migration across IL-1β-activated MC monolayers. A predominant role for IL-8 was found. We have previously shown that activated mesothelial monolayers are able to create a chemotactic gradient of IL-8, directed towards the abdominal cavity, through polarized secretion of IL-8.[9] A combination of mAbs of the two major cytokines TGF-β and IL-8, or the receptor antagonists against PAF showed, in contrast with endothelial cells,[5] no further inhibition of migration across mesothelial cells, above and beyond to the inhibitory effect of mAb against IL-8 alone.

Adherence of neutrophils to other cells is dependent on the family of β_2 integrin proteins (CD11/CD18), of which CR3 (CD11b/CD18) is known to be the most important leukocyte adhesion molecule involved in neutrophil adherence.[35, 36] The adhesion protein ICAM-1 found on activated endothelium is the best-known counter structure for CR3 in neutrophil adherence, but others may also exist.[37, 38] ICAM-2 is such a recently decovered counter structure for CR3, but is not expressed on resting or activated mesothelium.[7] Our results suggest that PMN adhesion to and migration across MC monolayers is, to a large degree a CD18/ICAM-1 mediated process. Antibody against CD18 was able to reduce the IL-1β induced neutrophil adherence and migration to levels observed with non-stimulated mesothelium. However, an exclusive role of ICAM-1 is questionable, since antibodies against ICAM-1 reduced the PMN migration across MC monolayer significantly, but incompletely. Furthermore, we found that ICAM-1 is already present in significant copy number on the surface of non-stimulated mesothelial cells, whereas PMN did not adhere to the surfaces of non-stimulated mesothelium. Moreover experiments with IL-1β stimulation of MC in the presence of actinomycin D showed that the surface expression of ICAM-1 per se is insufficient for neutrophil adherence. Thus, ICAM-1 surface expression did increase under these conditions but neutrophil adherence was not (Fig 2).

Qualitative alterations in affinity of adhesion receptors as an essential step in

neutrophil adherence has been described for the ICAM-1 receptor[39] as well as for the neutrophil CR3 receptor.[40-42] From our previous studies with endothelium it has become clear that an increase in the affinity of CR3 on PMN for its ligand on endothelial cells is required before they can adhere to endothelial surfaces.[4, 43] This increase in CR3 affinity can be induced either by soluble PMN stimuli (PMA, chemoattractants) or by interaction of E-selectin ELAM-1 on activated endothelial cells with SLe^x-bearing glycoproteins on the PMN.[4] Our present results indicate that neutrophil activation is also an important step in PMN adherence to MC monolayers. Indeed, PMA induced extensive neutrophil adherence, regardless of the activation state of the MC. Thus, neutrophils may bind to preformed ICAM-1 present on the surface of MC once the affinity of their CR3 is somehow boosted. Our Ca^{2+} flux experiments further support to this hypothesis. Co-incubation of IL-1β-activated MC with neutrophils was found to induce a rapid rise in $[Ca^{2+}]_i$ in the neutrophils. However, the neutrophil CR3 receptor seemed not to be directly involved in this effect since addition of mAb against CD18 did not alter the rapid rise in Ca^{2+} flux of the PMN (data not shown). Since we (this study) and others[7,44] have failed to detect E-selectin on either resting or activated MC, other MC surface proteins or products must be responsible for this process. Our present results strongly indicate that direct cell contact between the IL-1β-stimulated MC and the PMN activates the neutrophils. We further proved that protein synthesis during MC stimulation is required for activation of the neutrophil adherence to MC. We have excluded a possible role of MC-bound PAF, TGF-β and IL-8 in this process. Also, a role for selectins seems unlikely, because neuraminidase treatment of either cell type had no effect. Although our data suggest that a conformational change in CR3 is the essential step in increased neutrophil adherence to IL-1β-activated MC, direct evidence of such change should be forthcoming from further experiments.

From the data presented in this study it can be concluded that the mesothelium itself is actively involved in the process of transmesothelial cell migration of PMN, as observed in the inflammatory response during bacterial peritonitis.

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4

PERITONEAL INTERLEUKIN-8 IN ACUTE APPENDICITIS

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ABSTRACT

Interleukin-8 is a chemoattractant highly selective for neutrophils. This study was designed to investigate the presence of IL-8 in peritoneal fluid of patients with acute appendicitis. The clinical circumstances underlying the secretion of IL-8 by mesothelium and its mechanism of activation have not been defined. In an in vitro model for bacterial peritonitis the role of bacteria in activating human mesothelial cells to secrete IL-8, was studied. Cultured human mesothelium was incubated with various species of pathogenic bacteria, isolated from peritoneal exudate fluids of patients with appendicitis. The amount of IL-8 secreted by the cultured mesothelial cells was determined in an IL-8 ELISA, as was IL-8 present in the original peritoneal fluid of these patients. Peritoneal fluids from patients with a perforated appendix were found to contain a significantly higher concentration of IL-8 compared to peritoneal fluids from patients with a non-perforating appendicitis (121.6 (57.8) ng/ml versus 0.2 (0.07) ng/ml, respectively; mean (SEM), $p \leq 0.001$). Species of *Bacteroïdes* and *Fusobacterium necrophorum* induced IL-8 secretion from cultured mesothelial monolayers to levels comparable to those found in peritoneal fluids in vivo. Heat-killed bacteria and bacterial supernatant were also able stimulate mesothelium to secrete IL-8. The results suggest that in the early phase of bacterial peritonitis the influx of PMN is regulated by bacteria-induced IL-8 secretion by the mesothelium lining the peritoneal cavity.

INTRODUCTION

During bacterial peritonitis a massive influx of polymorphonuclear leukocytes (PMN) from the blood stream into the peritoneal cavity is observed. In vitro experiments have indicated that activated mesothelium takes part in this process by secreting interleukin-8 (IL-8), a chemoattractant for PMN.[1-3]

IL-8 is a small protein (8 kD) that belongs to the CXC-family of cytokines.[4] It is a chemoattractant highly selective for PMN, as compared with other chemotactic agonists. The effect of IL-8 on neutrophils is not restricted to the influx of PMN into inflamed tissue. At high concentrations it can also activate PMN to degranulate their intracellular stores,[4] thereby promoting the inflammatory reaction.

In vitro experiments with cultured human mesothelium have shown that IL-8 is secreted upon activation by IL-1β or TNF-α.[1-3] This mesothelial cell-derived IL-8 may account for the high IL-8 concentration in peritoneal effluent fluid found during continuous ambulatory peritoneal dialysis (CAPD)-related bacterial peritonitis.[5]

This study was designed to investigate the presence of IL-8 in peritoneal fluid of patients with acute appendicitis. In an in vitro model for bacterial peritonitis the role of bacteria in activating cultured human mesothelial cells to secrete IL-8, and its activating mechanism was studied.

MATERIALS AND METHODS

The study population comprised 23 patients with a histologically proven diagnosis of acute appendicitis, consisting of 16 patients with a non-perforated and 7 patients with a perforated appendix. Peritoneal fluid was obtained at the time of appendectomy. Free peritoneal fluid was aspirated into a syringe immediately on entering the abdominal cavity. Patients without free fluid were excluded from the study. An aliquot of the fluid was cultured and the remainder was centrifuged at 3000 rpm for 10 min. The supernatant was frozen and stored at -70°C until use for IL-8 assay.

Mesothelial cells (MC) were isolated from human omentum according to techniques modified from Nicholson et al.[6] and Wu et al.,[7] as described earlier.[8] In short, small pieces of omentum were removed early in the operative procedure from patients undergoing abdominal surgery for non-infectious conditions. All patients gave informed consent. The omentum was transferred to fluid containing 0.05% trypsin - 0.02% EDTA (Gibco, Life Technologies, Paisley, UK). After 15 min the detached MC were pelleted by centrifugation at 1200 rpm for 5 min and resuspended in supplemented M-199 (Gibco). MC were subcultured to confluent monolayers in 6-well culture dishes (Costar, Cambridge, MA). The identity of the MC was demonstrated by the absence of von Willebrand factor staining[9] and the presence of intracellular cytokeratins through immunofluorescence with monoclonal antibodies[10] (Dakopatts, Glosstrup, Denmark).

To investigate whether bacteria can directly induce human mesothelium to secrete IL-8, monolayers of human MC were incubated with the various bacterial species cultured from peritoneal fluid samples of the patients. IL-8 secretion induced by bacteria was compared with mesothelial IL-8 secretion induced by lipopolysaccharide (LPS) derived from *E. coli* (1 µg/ml; Sigma Chemical Co, St Louis, MO) and by the inflammatory cytokines interleukin-1β (IL-1β) (25 U/ml; Genzyme Co, Boston, MA) and tumor necrosis factor-α (TNF-α) (100 U/ml, Genzyme Co, Boston, MA), the latter two are very effective activators of MC.[3] The supernatants from MC incubated without a stimulus were used as controls.

Heat-killed bacteria were obtained by incubating 10^8 bacteria/ml in medium during 30 min at 60°C. Thereafter, the heat-killed bacterial suspension was added to the MC monolayer and co-cultured for 24 h. Control culture showed no residual bacterial growth. Bacterial supernatant was obtained by incubating 10^8 bacteria/ml in medium during 24 h at 37°C, followed by filtering the bacterial suspension through an 0.2-µm filter. Subsequently, the filtered bacterial supernatant was incubated for 24 h with MC monolayers. A sample of the filtered supernatant was cultured to confirm the absence of bacterial growth. Culture of the MC monolayers with and without IL-1β (25 U/ml; Genzyme Co.) served as positive and negative controls, respectively.

To study the effect of other bacterial species which were not isolated from the peritoneal fluid samples of patients with acute appendicitis, MC were also incubated with laboratory strains of bacteria at a final concentration of 10^8 bacteria per ml. Bacterial species included *Staphylococcus aureus* Cowan 1 and *Staphylococcus aureus* Wood 46 which have been described in other studies.[11, 12] *S. epidermidis* V72 and *S. epidermidis* V10 were clinical strains isolated from patients with CAPD-related bacterial peritonitis.

After 24 h incubation with either bacterial supernatant, heat-killed bacteria, or intact bacteria, the supernatant of the mesothelial cell monolayer was collected, centrifuged at 3000 rpm for 10 min and stored at -70°C until use for IL-8 assay.

All bacterial species were allowed to grow under appropriate microbiological aerobic or anaerobic conditions prior to incubation with the MC monolayers. The medium used for the bacterial incubation with the monolayers contained no antibiotics. All bacterial species were able to grow rapidly in this medium during 24 h as confirmed by densitometry. However, the strain of *Fusobacterium necrophorum* showed minimal growth under these relatively aerobic conditions. In the absence of MC no IL-8 was detected in medium containing bacteria. There was a wide range of morphological toxic effect of different bacteria to the MC monolayers during the 24 hour incubation; no correlation could be found between this toxic effect and ability or disability of the MC to secrete IL-8.

The concentration of IL-8 in the peritoneal fluid samples and in the supernatants of MC cultures was determined with a previously described IL-8 ELISA.[13]

The Mann-Whitney test was used for statistical analysis of IL-8 data obtained from the peritoneal fluids; the unpaired *t* test was used for statistical analysis of the IL-8 secretion by the cultured MC.

RESULTS

The level of IL-8 in the peritoneal fluid of patients with acute appendicitis varied significantly between the perforated versus non-perforated status of the inflamed appendix ($p \leq 0.001$, Figure 1). In the patients with a non-perforated appendix (n=16) IL-8 was undetectable in 8 patients and only low concentrations of IL-8 (≤ 1 ng/ml) were measured in the remaining 8 patients.

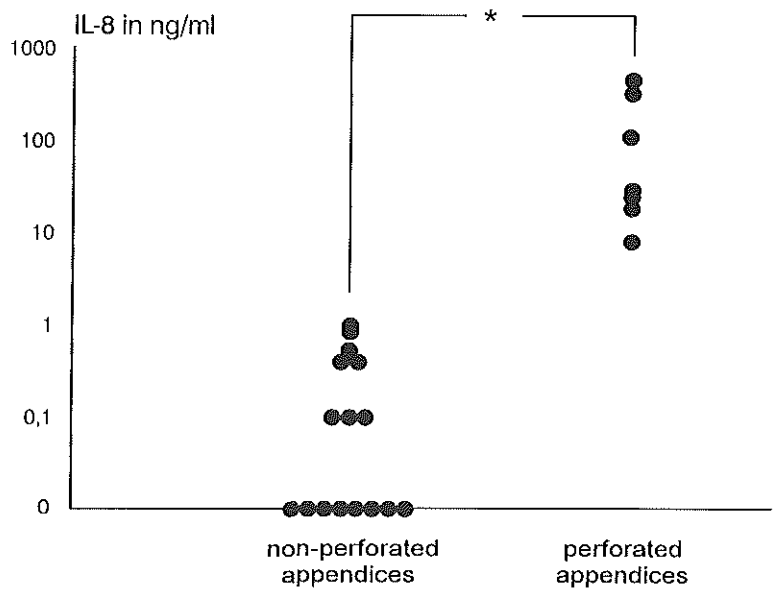

Figure 1. Peritoneal interleukin-8 levels in patients with non-perforated (n=16) and perforated (n=7) acute appendicitis; * $p \leq 0.001$.

In sharp contrast, all samples of patients with a perforated appendix (n=7) contained IL-8 concentrations at much higher concentrations, ranging from 7.6 ng/ml to 377.3 ng/ml with a mean (SEM) value of 121.6 (57.8) ng/ml (Figure 1). The IL-8 concentration in endoluminal appendix fluid of inflamed but non-perforated appendices contained low concentrations of IL-8 (data not shown). It is therefore unlikely that the high concentrations found in the peritoneal fluid samples after perforation of the appendix originate from IL-8 produced in the gastro-intestinal tract.

All cultures of peritoneal fluid from patients with a non-perforated appendicitis remained sterile, whereas all samples of patients with a perforated appendix grew, usually multiple, species of bacteria. To investigate the relationship of bacteria and IL-8 in the peritoneal fluid of patients with perforated appendicitis, peritoneal MC were incubated in vitro with the bacteria isolated from patients with perforated appendicitis. The IL-8 concentrations found in the supernatant of MC monolayers after 24 h of incubation with these bacterial species are listed in Figure 2. Heat-killed bacteria and supernatant obtained from the bacteria isolated from patients with perforated appendicitis were also able to activate mesothelium to secrete IL-8.

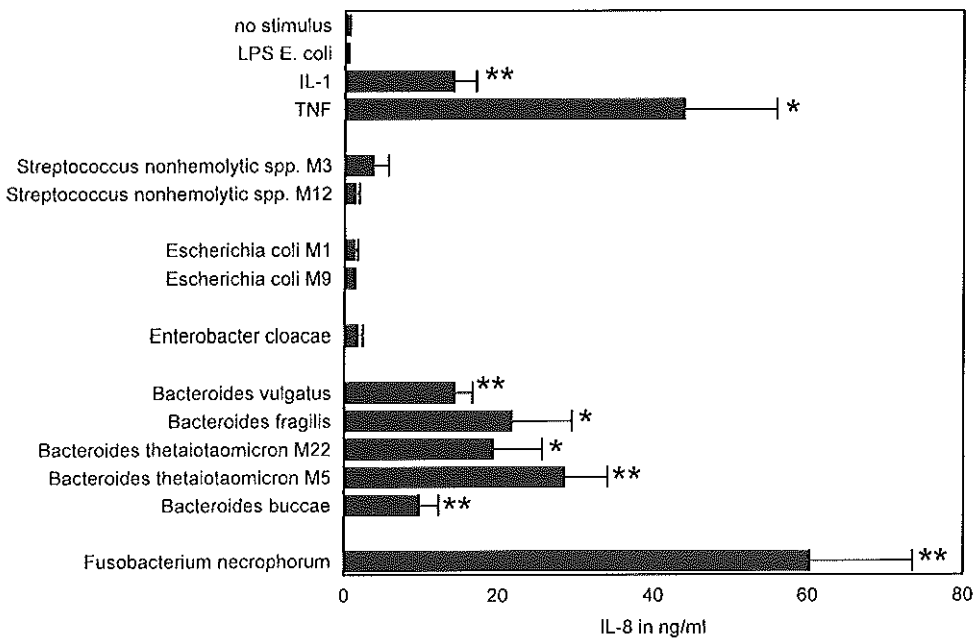

Figure 2. Interleukin-8 secretion by human mesothelial cell monolayers after 24 h incubation with bacteria (10^6/ml), cultured from peritoneal fluid samples of patients with perforated appendices. Results represent the mean (SEM) of three to four separate experiments. * $p \leq 0.05$, ** $p \leq 0.01$.

An example is shown for *Bacteroïdes fragilis* in Figure 3. All other cultured bacteria which were able to stimulate mesothelium, showed the same pattern of IL-8 secretion (data not shown).

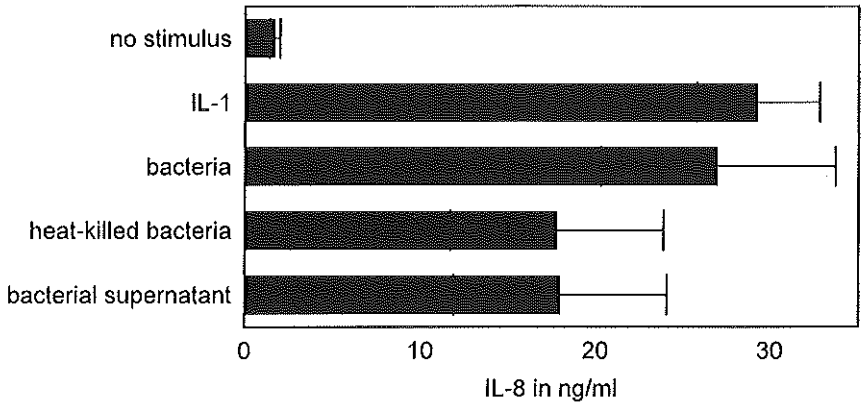

Figure 3. Interleukin-8 secretion by human mesothelial cell monolayers after 24 h incubation with *Bacteroïdes fragilis*, heat-killed bacteria and bacterial supernatant of the same bacterial species. Results represent the mean (SEM) of three separate experiments. The difference in IL-8 secretion after incubation with heat-killed bacteria or bacterial supernatant, compared to intact bacteria, was not significant.

There was a wide variation between bacterial species in their ability to activate human MC to secrete IL-8 in vitro. All tested *Bacteroïdes* isolates induced MC to secrete significant amounts of IL-8. Remarkably, the effect of *Fusobacterium necrophorum* even surpassed the IL-8-inducing effect of an optimal concentration of TNF-α on mesothelium (Figure 2). In contrast, *Escherichia coli*, LPS derived from *E. coli* and *Streptococcus spp.* were poor inducers of mesothelial IL-8 secretion in this setting (Figure 2).
The activating effect of bacteria on MC was not restricted to anaerobic gram-negative bacteria, because *Staphylococcus epidermidis* and *Staphylococcus aureus* were also able to induce mesothelium to produce and secrete IL-8 (Table 1).

DISCUSSION

Bacterial invasion of the peritoneum often causes severe inflammation, characterized by a strong influx of neutrophils into the peritoneum. Cytokines are important mediators of this inflammatory process. Until recently it wasbelieved that during inflammation activated monocytes are the main source of the cytokine IL-8,[14] one of the most potent and selective chemoattractants for neutrophils.[4] However, in vitro models of peritonitis have

Table 1. Interleukin-8 secretion by human MC monolayers after 24 h incubation with laboratory strains of common pathogenic bacterial species, frequently causing CAPD-related peritonitis.

	IL-8 in ng/ml		
No stimulus	0.6	(0.1)	
S. aureus Cowan 1	14.9	(2.8)	**
S. aureus Wood 46	1.8	(0.7)	
S. epidermidis V72	26.2	(7.9)	*
S. epidermidis V10	10.9	(1.4)	**

Table 1. IL-8 is expressed as the mean (SEM) of four separate experiments, compared to no stimulus. * $p \leq 0.05$, ** $p \leq 0.01$.

shown that human mesothelium, if properly stimulated, is also capable of producing IL-8.[1-3]

It has been suggested that the infected peritoneum may be the primary source of increased levels of cytokines, circulating in the bloodstream.[15, 16] In the present study it was shown indeed that anaerobic bacterial species, commonly found in peritoneal fluid during surgical peritonitis[17] can induce human mesothelial monolayers to secrete large amounts of IL-8. This phenomenon suggests that the high concentrations of IL-8 found in peritoneal fluid of patients with perforated appendicitis is due to bacteria-induced secretion of IL-8 by the mesothelium. This finding concurs well with the observed physiologic course of inflammation during peritonitis: a massive influx of neutrophils into the peritoneal fluid is usually observed in the early phase of peritonitis, followed within 24 h by the migration of large numbers of monocytes.[18]

Other evidence for local production of the cytokine IL-8 is provided by a study of Agace et al.,[19] in which in an experimental model of urinary tract infection (UTI), women were purposely colonized in their urinary tracts with uropathogenic *Escherichia coli*. An urinary IL-8 response was found in all women, but no serum IL-8 was detected, suggesting that urinary tract epithelial cells are the primary source of IL-8 production during UTI. Other examples of bacteria-induced IL-8 secretion were published recently by Crabtree et al.[20] and Massion et al.[21] in which the increase in IL-8 secretion by neoplastic gastroduodenal mucosa or airway epithelial cells was found to be associated with *Helicobacter pylori* and *Pseudomonas* infection, respectively.

The ability of bacterial supernatant and heat-killed bacteria to activate MC monolayers to secrete IL-8 suggests that neither bacterial adhesion to MC nor living bacteria are required for IL-8 secretion. Furthermore, the activating

capacity of bacterial supernatant shows that bacteria are able to secrete one or more compounds that can activate MC. This concurs with previous findings that a heat-stable product of *Pseudomonas*, resistant to inactivation by trypsin or pronase, with a molecular mass smaller than 1 kD, was responsible for IL-8 secretion by airway epithelial cells.[21]

Our results show that induction of mesothelial IL-8 secretion by bacteria is to some extent species specific. Further research will be necessary to delineate which bacterial factors induce the mesothelium to secrete IL-8. There may even be a tissue specificity for each species of bacteria in IL-8 induction, because *Escherichia coli*, which was shown to stimulate human epithelial urinary tract cells to secrete IL-8 profoundly,[19] did not have any effect on IL-8 production by human MC (this study), airway epithelial cells[21] or fibroblasts.[22]

The wide range of IL-8 secretion found in the peritoneal fluid samples of the patients with a perforated appendix (7.6 - 377.3 ng/ml) can probably be explained by the variation in the ability of different bacterial species to stimulate MC to secrete IL-8, and by the variable densities at which they were present in the abdominal cavity.

Induction of IL-8 secretion by MC was not restricted to anaerobic gram-negative bacteria, since *S. aureus* and *S. epidermidis* were also able to induce mesothelial IL-8 secretion. Thus, this route of mesothelial cell activation may not only occur in surgical but also in other types of peritonitis, such as those seen during CAPD related peritonitis, which is frequently caused by these staphylococci.

We have previously shown that activated mesothelial monolayers are able to create a chemotactic gradient towards the abdominal cavity, through polarized secretion of IL-8,[23] which induces a profound migration of PMN across activated mesothelial monolayers.[23] The ability of different bacteria species to induce human mesothelium to secrete IL-8, and thereby to promote a neutrophil influx into the peritoneal cavity may be of clinical relevance. The neutrophil influx contributes to the severity of the inflammatory reaction.[19] Neutrophils possess a highly destructive potential, together with little intrinsic ability to differentiate between foreign and host antigens. These cells are therefore implicated as mediators of tissue destruction in inflammatory states.[24] It might be that an overshoot of the neutrophil influx plays a role in the severity of peritonitis. It will be interesting to study in peritonitis, together with antibiotics, the use of therapeutics capable of modulating the neutrophil influx into the peritoneal cavity, e.g. by blocking IL-8 through antibody infusion into the peritoneal cavity.

Our results indicate that the early phase of bacterial peritonitis is a localized process in which various bacterial species are able to interact with human mesothelium and induce these cells to secrete large amounts of IL-8, thus

triggering the inflammatory cascade.

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5

OPSONIN TRANSPORT ACROSS CULTURED HUMAN PERITONEAL MESOTHELIUM

Influence of polymorphonuclear leukocyte transmigration

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ABSTRACT

We studied the transport of opsonins across monolayers of cultured human mesothelial cells. Also, the role of PMN migration on the transport of opsonins was investigated.
The opsonic activity was measured functionally through the uptake of preopsonized bacteria by the PMN. Mesothelial cells were isolated from human omentum and cultured on Transwell filters to create a confluent layer of cells.
Mesothelial cell monolayers are a strong, but not absolute, barrier to the diffusion of opsonins. PMN migration across the mesothelial monolayers increases the rate of opsonin transport, which could be modulated by CD18 specific antibodies or by omitting the chemotactic agent FMLP.
We show that a monolayer of human peritoneal mesothelial cells forms an effective barrier against opsonin transport, and that this barrier is breached during inflammation primarily due to PMN migrating across this monolayer.

INTRODUCTION

Opsonisation of bacteria and other particles greatly enhances their recognition and subsequent uptake by phagocytic cells. Deficiencies in the major serum opsonic molecules, IgG and C3, are well known to predispose patients to bacterial infection. Such a state of opsonic deficiency exists locally in the peritoneal cavity of patients undergoing chronic ambulatory peritoneal dialysis (CAPD) for end-stage renal disease.[1-3] Only strains of bacterial species such as *Staphylococcus epidermidis*, which are opsonized at relatively low serum concentrations (well below 1-2%), can be properly opsonized upon incubation in the effluent peritoneal dialysis fluid from CAPD patients. However, even then, many effluent fluids lack sufficient opsonic 'power' to opsonize a given strain of *S. epidermidis*.[1] Moreover, the heterogeneity in strains of *S. epidermidis* is such that many require opsonic concentrations that are simply not available in the effluent fluids of most uninfected CAPD patients. [4]Little is known about the diffusion or 'leakage' of opsonins into effluent fluids. It has been shown that the opsonic power gradually increases with increasing dwell times.[2] Also, during episodes of peritoneal inflammation, the exudation of inflammatory cells - predominantly polymorphonuclear leukocytes (PMN) - is accompanied by increased loss of serum proteins into the dialysate, which thus enhances the opsonic power of the effluents. Because the peritoneal membrane is the obvious barrier against access to the peritoneal cavity, we have studied the transport of opsonins in an in-vitro model system that mimics the peritoneal membrane.

MATERIALS AND METHODS

CAPD Effluents

Serial effluents from a 6-8 h dwell time were collected from 11 CAPD patients followed over time. For each patient 2-6 effluent samples were selected from infection-free intervals. These samples proved to be sterile upon culture and contained less than 10^7 cells/l effluent. Also, from each patient 1-3 effluent samples were studied that were collected in the acute phase of peritonitis. These samples all contained $\geq 10^9$ cells/l effluent and all but one grew a bacterial pathogen. All effluents were centrifuged and the cell-free supernatants were stored at -70°C until used in the opsonisation assay. The IgG content of each sample was determined by radial immuno-diffusion (LC-Partigen-IgG, Behringwerke AG, Marburg, Germany).

Bacteria and radiolabeling

Throughout the study a clinical isolate of *S. epidermidis* was used. For each experiment colonies from bloodagar were suspended in Mueller-Hinton broth containing 20 µCi (400 KBq) [8-^{3}H] - adenine (Amersham, Buckinghamshire, UK) and were incubated overnight. The bacteria were subsequently washed free of excess radiolabel, were resuspended in Hanks Balanced Salt Solution containing 0.1% gelatin (GHBSS; Gibco, Life Technologies, Paisley, UK) to a final concentration of 5×10^8 CFU/ml and were stored on ice until use.

Opsonization procedure

Ten µl of radiolabeled bacteria were mixed with 100 µl of indicated effluent, or Transwell culture fluid (*vide infra*), and were incubated for 30 minutes at 37°C. Opsonization was stopped by adding 2.5 ml of ice-cold phosphate-buffered saline, pH 7.4 (PBS) to the wells; the bacteria were then pelleted (15 min at 1600 g) and resuspended in 100 µl of fresh GHBSS (preopsonized bacteria, 5×10^7 CFU/ml). Bacteria were likewise preopsonized with human pooled serum (HPS) at indicated concentrations, and with effluents or serum that, prior to use, had been heated (30 min at 56°C) to inactivate their heat-labile opsonic activity.

Determination of opsonic activity

The opsonic activity of effluents, in vitro culture fluid and HPS was determined by measuring the uptake of preopsonized bacteria by human polymorphonuclear leukocytes (PMN) in an assay that has been described in detail.[4] PMN were isolated from venous blood of healthy donors and used at 5×10^6 cells/ml GHBSS. 100 µl of preopsonized bacteria was mixed with 100 µl of PMN suspension in a final bacteria to phagocyte ratio of 10:1. Phagocytosis was allowed to proceed for 12 min in a shaking incubator at 37°C. Phagocytosis was stopped by adding 2.5 ml of ice-cold PBS. Non-phagocyte-associated bacteria were removed by three cycles of differential centrifugation (5 min at 160 *g*). The PMN-associated radioactivity in the final pellet was determined by liquid scintillation counting. Phagocytosis was expressed as the percentage of total radioactivity added, determined in a separate vial, and this uptake was used as a measure of opsonization. The opsonin-dependency of *S. epidermidis* uptake by PMN was carefully determined by preopsonization in serial dilutions of HPS and heated HPS. Figure 1 shows that the test strain of *S. epidermidis* required ≤1% HPS to become opsonized for uptake by human PMN. Without serum, uptake was always ≤5%. Opsonization with heated serum was about half as effective as that found with fresh serum (Figure 1).

Mesothelial cell monolayers

Mesothelial cells (MC) were isolated from human omentum as described previously.[5] To study the transport of opsonins across mesothelium, the MC were subcultured in medium M-199 (Gibco) to confluent, inverted monolayers on polycarbonate membranes (8.0 µm pore size) of Transwell cell culture chamber inserts (Costar no. 3422, Cambridge, MA), as recently described.[6] The MC monolayers

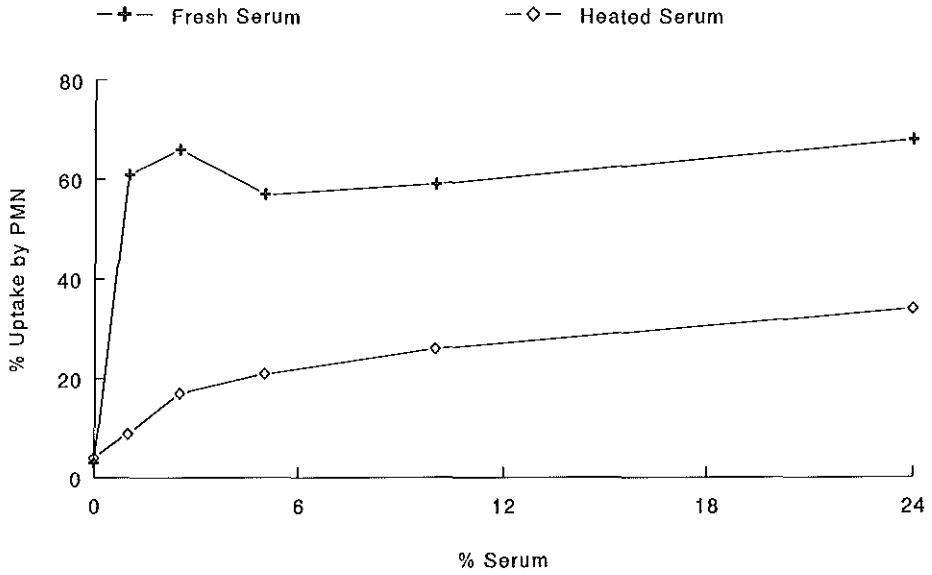

Figure 1. Opsonic requirements of the test strain of S. epidermidis for uptake by human PMN. Bacteria were preopsonized with the indicated concentrations of fresh or heat-inactivated pooled human serum and were subsequently mixed with human PMN. % uptake was determined after 12 min of incubation at 37°C.

reached confluency in 5 days, as determined by phase-contrast microscopy, by microscopy of May-Grünwald/Giemsa stained filters and by electrical resistance measurements. Each monolayer was visually tested for unwanted leaks by placing it in a well with culture medium for several minutes; only filters that did not show accumulation of medium above the filter were accepted for the experiments.

Experimental design

In all experiments the transport of serum opsonins was tested by adding 50% HPS in culture medium to the upper compartment of an MC monolayer-containing chamber insert and placing it in a well containing cell culture medium. After incubation at 37°C for specified times, the fluid in the lower compartment was harvested, centrifuged, and the cell-free supernatant was stored at -70°C until tested for its opsonic activity (*vide supra*). From the volume ratios of the upper *versus* the lower compartment, the HPS concentration in the upper compartment and the opsonic requirement of the test strain of *S. epidermidis*, it was estimated that the system would 'sense' the transport of ≥5% of the *S. epidermidis* opsonic activity added to the upper compartment, i.e. it was highly sensitive in detecting opsonins transported to the lower compartment. The effect of PMN chemotaxis across the MC monolayers on the transport of opsonins was tested by adding human PMN at specified concentrations to the upper compartment of the chamber insert, in the presence of 50% HPS and by adding M-199 with or without 10^{-8} M formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma Co., St Louis, MO) in the lower compartment. PMN migration under these conditions was always ≥15% in 30 min in the presence of FMLP and ≤5% in its absence.[6] In some experiments PMN and MC monolayers were preincubated for 15 min with monoclonal antibody CLB-LFA-1 (IgG_1), which recognizes the CR3 β-chain CD18 and blocks CR3's adherence mediating function.[7]

RESULTS

Opsonins in effluents

From 11 studied CAPD patients, the opsonic activity and total IgG content of their effluents were tested when they were completely free of peritonitis and when they experienced clinical peritonitis. Effluents from infection-free intervals were only used when they contained $\leq 1x10^7$ cells/L, with < 5% PMN, to assure the non-infected state of the peritoneal cavity. For each patient the average *S. epidermidis* opsonic activity and IgG concentration, based on 2-6 such effluents, was determined. Likewise, the opsonic activity and IgG content were determined during the acute phase of bacterial peritonitis of effluents that contained $>10^9$ cells/ml. In all patients, their effluent IgG concentration as well as its heat-labile and heat-stable opsonic activity was higher during peritonitis compared to the infection-free intervals. However, the variation in these parameters was considerable, especially during episodes of peritonitis (Table 1). In 4 patients, low opsonic titers during peritonitis were associated with relatively low levels of total IgG in their effluents at that time (Table 1; patient nr. 8-11).

Table 1. *S. epidermidis* opsonic activity and total IgG content of effluent from CAPD patients during infection-free intervals and at the time of peritonitis.

	Opsonic activity fresh effluent		Opsonic activity heated effluent		IgG Content	
Peritonitis	*Without*	*With**	*Without*	*With**	*Without*	*With**
Patient nr.						
1	7	78	7	68	120	430
2	30	74	30	43	63	430
3	18	43	13	23	110	475
4	13	76	7	34	106	365
5	7	64	6	45	70	350
6	16	65	15	53	130	460
7	9	48	9	28	50	447
8	8	24	6	15	20	150
9	5	28	4	9	25	150
10	23	37	17	35	106	190
11	22	36	13	20	133	250

Table 1. Bacteria were preopsonized in fresh or heated effluent and their uptake by PMN was subsequently measured and expressed in % uptake. The total IgG was determined by radial immuno diffusion and expressed in mg/l. The opsonic activity and the total IgG content was significantly higher during peritonitis, *$p \leq 0.01$, paired *t* - test.

Mesothelium as an opsonic barrier

Because opsonins have to cross the peritoneal membrane en route to the peritoneal cavity, the MC monolayer may constitute a significant barrier in their pathway. Such a barrier to opsonin transport was indeed shown to exist in an in vitro double-chamber culture system in which a confluent MC monolayer attached to a porous polycarbonate filter separated the two compartments. As can be seen in Figure 2, bare filters (pore size 8.0 µm) constituted only a very minor barrier to the diffusion of serum opsonins from the upper to the lower compartment of the test system. Sufficient heat-labile opsonic activity for detection was present in the lower compartment within 30-60 min incubation. Heat-stable opsonins were also detected within 60 min. This activity continued to gradually increase over the 24-hour incubation time (Figure 2). Filters covered with a confluent layer of MC showed that transport of opsonic molecules occurred at a much slower rate, without much activity detectable in this lower compartment within the first 4 h of incubation. However, at 24 h opsonic activity of the medium in the lower compartment was comparable to that of chambers with bare filters. The same pattern of transport across MC monolayers was found for IgG (data not shown).

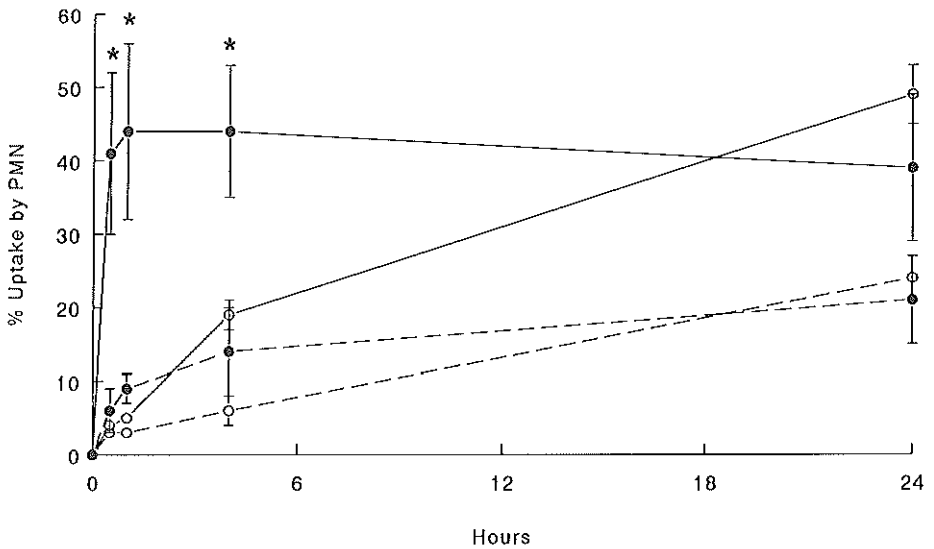

Figure 2. Transport of serum opsonic activity across bare 8.0 µm Transwell filters (closed dots) and across the same type of filter with a confluent layer of human mesothelial cells (open dots). Transport of serum opsonins was determined at indicated intervals after addition at time zero of 50% pooled human serum, either fresh (straight lines) or heat-inactivated (dotted lines), to the upper compartment by sampling the lower compartment of the Transwell culture system. Opsonic activity was determined in a PMN phagocytosis assay (see Materials and Methods). Data are means ± SEM based on at least 3 separate experiments performed in duplicate. *Significant higher uptake compared to the uptake across filters with a confluent layer of human MC, $p \leq 0.01$, paired *t* - test.

Thus, the mesothelial cell monolayer constituted a strong, but not absolute, barrier to the diffusion of opsonins and IgG in this double-chamber assay.

Influence of PMN diapedesis

Because inflammation of the peritoneal cavity involves the diapedesis of large numbers of PMN from the capillary bed across the peritoneal membrane, and is associated *in vivo* with a concomitant increase in the opsonic activity of the peritoneal fluid, we studied the effect of PMN migration perse on the transmesothelial transport of opsonins in the double-chamber culture system. To mimic the *in vivo* situation, PMN were added to the upper compartment of the Transwell system containing 50% HPS and were enticed to migrate through the filter (and its adherent MC layer) towards the chemotactic agent FMLP. We found that, in a dose-dependent manner, the migration of PMN through the mesothelium was associated with an increase in the rate of opsonin transport (Figure 3) and IgG transport (data not shown) across the filter to the lower compartment. However this effect was not significant (paired *t*-test). To further test the hypothesis that PMN diapedesis across mesothelium induces opsonin transport we studied opsonin transport in the presence of non-migrating cells in the upper compartment.

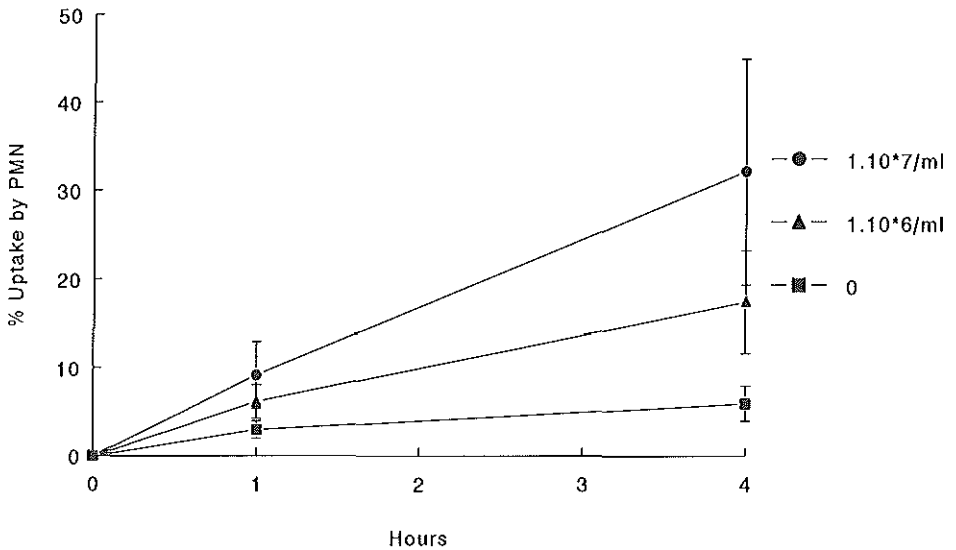

Figure 3. Dose-response effect of PMN migration across MC monolayer on the transport of opsonins. PMN at indicated concentrations and opsonins (50% HPS) were added to the upper compartment and 10^{-8} M FMLP was added to the lower compartment. At the indicated times, the fluid in the lower compartment was tested for opsonic activity in the PMN phagocytosis assay with radiolabeled *S. epidermidis* (see Materials and Methods). Data are means ± SEM based on at least 3 separate experiments performed in duplicate.

Non-migration was achieved either by omitting the chemotactic agent FMLP from the lower compartment or by blocking the PMN CR3 receptor. This latter receptor is needed for PMN adherence to and migration across MC, and can be blocked to a large extent by specific antibodies to the β-chain (CD18) of the CR3. As a further control, the potential action of the chemotactic agent alone on the transmesothelial transport of opsonins was also studied. The data show that PMN migration is indeed needed to obtain the enhanced opsonin transport and that FMLP by itself is not sufficient to elicit this phenomenon (Figure 4).

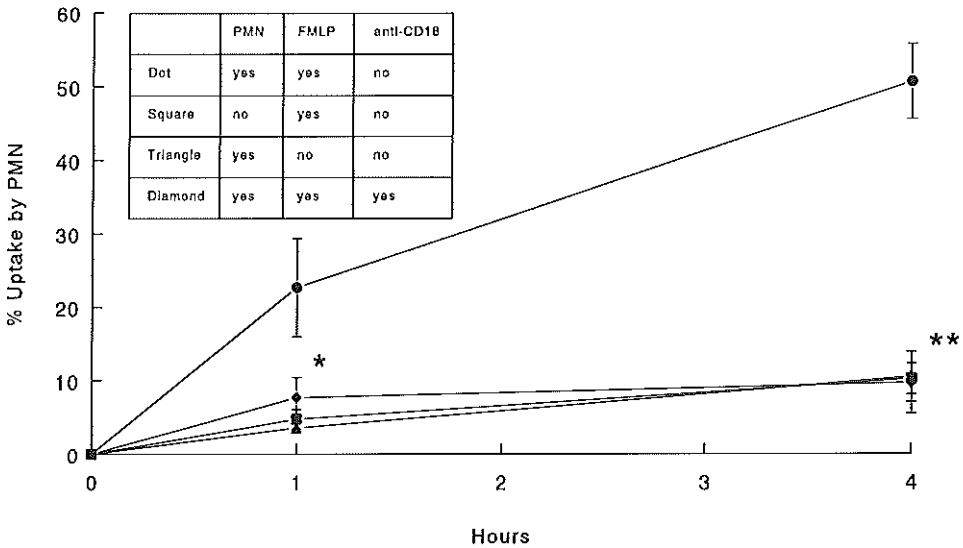

Figure 4. Transport of *S. epidermidis* opsonins across human MC monolayers in the presence or absence of PMN (10^7/ml), FMLP (10^{-8} M) and monoclonal antibody against PMN membrane CD18 (CR3). Indicated combinations were added at time 0 and opsonin transport was measured in samples taken at 1 and 4 h by the PMN phagocytosis of *S. epidermidis* assay (see Materials and Methods). Data are means ± SEM based on at least 3 separate experiments performed in duplicate. The absence of PMN, FMLP or the presence of CD18 antibodies gave a significant inhibitory effect on the opsonin transport, $^*p \leq 0.05$, $^{**}p \leq 0.01$, unpaired *t* - test.

DISCUSSION

The results of this study show that a confluent monolayer of human MC constitutes a strong barrier against the free flow of serum opsonins. Moreover, this barrier is, at least partially, breached when large numbers of inflammatory cells migrate through the monolayer. The movement of opsonic molecules, primarily IgG and complement factors, from the bloodstream to the peritoneal cavity is of utmost importance for the host defence at that site. It is well known, and was confirmed in this study, that the opsonic activity of non-infected CAPD

effluent is rather poor.[1-3] This is also true for the ascitic fluid accumulating in patients with liver cirrhosis,[8,9] which puts these patients at increased risk of infection. In CAPD patients, dialysate opsonic activity slowly increases with the dwell time, so that after 6-8 hours it approximates 1% of that of normal serum when assayed with *Staphylococci*.[2,10] Loss of opsonic protein into the dialysate is part of the total protein loss observed via spent dialysates during CAPD. These protein losses increase significantly during the acute phase of peritonitis in these patients.[11, 12]

Our observations suggest that the MC layer of the peritoneal membrane is an important barrier to the diffusion of opsonins and probably to other serum proteins as well. There may be several explanations for the observed increase in opsonic transport and consequent protein loss into the dialysate during peritonitis. All probably involve the integrity of the confluent monolayer of MC. The integrity of this cell layer is apparently lost to some extent as a consequence of the diapedesis of large numbers of PMN through the membrane during the inflammatory response. Others have shown a loss of electrical resistance across a human intestinal epithelial cell line (T84) when PMN migrate through this cell-layer, an event that also involves the PMN CR3.[13] On the other hand, removal of the mesothelial cell layer by physochemical damage also leads to increased loss of proteins into the peritoneal cavity.[12] Vasodilatory agents, including histamine and nitroprusside produce similar findings, i.e. direct loss of proteins at the site of application.[12] However, it is not clear whether the MC layer plays a role in these latter phenomena. We hypothesize that during acute bacterial peritonitis the migration of massive numbers of PMN and other inflammatory cells through the MC layer by itself may account for the observed concomitant effusion of serum opsonins (and other proteins) into the peritoneal cavity. Because, with our increase in knowledge about the inflammatory response, PMN migration may become more amenable to medical modulation. In the near future opsonin concentration at the site of infection and protein loss during peritonitis are likely be controlled as well.

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6

ACTIVATION STATE OF NEUTROPHILS ISOLATED FROM PERITONEAL EFFLUENT IN CAPD-ASSOCIATED PERITONITIS

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ABSTRACT

The activation of neutrophilic granulocytes (PMN) from the peritoneal effluent in chronic ambulatory peritoneal dialysis (CAPD)-associated bacterial peritonitis was investigated during the initial stage. The change in surface expression of membrane antigens was tested by FACS analysis. Chemotaxis of the neutrophils towards the neutrophil chemoattractants IL-8, PAF, C5a or FMLP was measured in a Boyden chamber assay. CD10, an 'early activation' marker on neutrophils, was upregulated on effluent PMN, as were CD11b, CD11c, CD45 and CD66b. The expression of CD63, a specific marker for azurophilic granules, had not changed and L-selectin was shed from the effluent *versus* peripheral neutrophils. Decreased chemotaxis in response to IL-8 and PAF was measured with effluent *versus* peripheral neutrophils, perhaps due to desensitization during the migration of PMN across endothelium and mesothelium. However, these changes were not significant. The effluent PMN were not apoptotic. Thus, during the initial stage of CAPD-associated peritonitis, effluent neutrophils have actively changed their marker profile. Also, a modest modulation of chemotactic responsiveness of the dialysate neutrophilic granulocytes is observed towards IL-8 and PAF.

INTRODUCTION

Neutrophilic granulocytes (polymorphonuclear leukocytes, PMN) are the initial cells infiltrating in areas of tissue inflammation.[1] Diapedesis of neutrophils from the circulation into inflammatory sites requires neutrophil adherence to and migration across the endothelial lining of the blood vessels. In the case of peritonitis, the neutrophils not only have to migrate across an endothelial cell layer when leaving the circulation but also across a mesothelial monolayer before entering the peritoneal cavity. Several in vitro studies have been performed to elucidate the role of activated endothelium[2, 3] and activated mesothelium[4] in PMN adherence to and migration across these cell layers. The present study investigates the combined role of endothelium and mesothelium in activating the neutrophilic granulocytes in vivo during episodes of peritonitis. The influx of PMN during peritonitis was studied in patients on continuous ambulatory peritoneal dialysis (CAPD) for chronic renal failure.[5]

Peritonitis remains a frequent complication of CAPD despite improvements of technique.[6] Peritoneal macrophages are the predominant phagocytic cell type found in the peritoneal effluent of uninfected CAPD patients.[7] However, with the development of clinical peritonitis, PMN rapidly enter the peritoneal cavity

and quickly become the dominant cell type in the effluent fluid.[8-10] An advantage of studying this type of peritonitis is the possibility to investigate the PMN in a standardized protocol at the very beginning of the inflammatory process, by isolating PMN from the first cloudy effluent. The direct influence of micro-organisms on PMN function is minimal in CAPD peritonitis, because the concentration of bacteria is usually less than 100 cfu/ml.[11]
Our study examines the difference in state of activation of peripheral blood PMN *versus* dialysis effluent PMN of the same patient at the initial stage of peritonitis, compared to control neutrophils of the peripheral blood of healthy donors. For this purpose, we investigated the changes in surface expression of adhesion receptors and of antigen markers representing the release of azurophilic or specific granules from neutrophils.[12] The degree of apoptosis of the PMN under these conditions was measured with annexin V.[13] We further investigated the chemotaxis of the PMN in response to various chemoattractants in a Boyden chamber assay.

PATIENTS AND METHODS

Reagents

Formyl-methionyl-leucyl-phenylalanine (FMLP), platelet activating factor (PAF), and C5a were purchased from Sigma Chem. Co., St. Louis, MO. IL-8 was purchased from Boehringer Mannheim, Mannheim, Germany. The basic incubation medium for cell suspensions contained 132 mM NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 6 mM KCl, 1.2 mM KH_2PO_4, 20 mM HEPES, 5.5 mM glucose, and 0.5% (w/v) HSA, pH 7.4.

mAb

The following mAb were used. Irrelevant murine control mAb of the IgG_1 and IgG_{2a} subclass were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands. CLB-LFA-1/2 (IgG2b),[14] CLB-B2.12 (IgM isotype)[15] and S-HCL3[16] are directed against the α-chains of CR3, LFA-1 and p150,95, respectively. CLB-LFA-1/1 (IgG1) recognizes the common β-chain CD18 of the β_2 integrins.[17] The other mAb used are directed toward CD10 (VIL-A1),[18] CD16 (FcRIII: CLB-FcR-gran1),[19] CR1 (CD35; D3J3),[20] CD45 (3D3, 15D9) (R.A.W. van Lier, CLB, Amsterdam, The Netherlands, personal communication), CD63 (435)[21, 22] and CD66b (formerly called CD67) (B13.9).[23] CD32 (FcRII; IV.3) and CD64 (FcRI; 32.2) were obtained from Medarex, West Lebanon, NH. Leu-8, against L-Selectin[24] was obtained from Becton Dickinson, Mountain View, CA.

Granulocyte isolation

Patients with end-stage renal failure who were on CAPD, participated in the study. Informed consent was obtained from all patients. Neutrophils were purified from the first cloudy dialysate of the patients, who developed peritonitis. Within three hours of discovering the cloudy effluent, 30 ml of citrate blood was obtained from the same patient. Control neutrophils of screened healthy blood donors were obtained likewise. Purification of all neutrophils started directly after obtaining blood and effluent samples. The blood neutrophils of patients and healthy volunteers were purified as described earlier.[25] In short, blood cells were separated by density gradient centrifugation over isotonic Percoll (Pharmacia, Uppsala, Sweden) with a specific gravity of 1.076 g/ml. The interphase, containing the mononuclear cells, was removed. The pellet fraction, containing erythrocytes and

granulocytes, was resuspended for 10 min with ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4) in order to lyse the erythrocytes. The remaining granulocytes were washed twice with PBS containing 0.5% HSA (w/v). Granulocytes were resuspended in the incubation medium at a final concentration of 10^6 cells/ml. Purity of the granulocytes was ≥98%, with ≥95% neutrophils. Viability measured by lactate dehydrogenase (LDH) release[26] was ≥95%. The neutrophils of the dialysate fluid were purified by density gradient centrifugation over isotonic Percoll as described above except that the pellet fraction was not treated with NH_4Cl solution but was washed twice with PBS containing 0.5% HSA and was resuspended in incubation medium for cell suspensions. It is known from a previous study[12] that the treatment of PMN with NH_4CL solution does not influence the activity state of the neutrophils. A second sample of peripheral blood was obtained from each of the patients at least three weeks after full recovery from peritonitis, i.e. when the patient had no symptoms and had clear effluents with less than 100 WBC/mm^3. The activation state of these neutrophils was again compared to neutrophils isolated from peripheral blood of healthy volunteers. The same purification method of the neutrophils was used.

Immunofluorescence flow cytometry

The expression of surface antigens of neutrophils was measured by indirect immunofluorescence with flow cytometry. In short, after addition of the primary mAb, the cells were incubated for 30 min at 4°C and were subsequently washed twice with ice-cold PBS, containing 0.5% (w/v) BSA. Thereafter, fluorescein-labeled goat-anti-mouse-Ig was added and incubated with the cells for another 30 min at 4°C. After two more washes, mAb binding was quantified for 10,000 cells with a FACScan (Becton Dickinson, Mountain View, CA) and was expressed as Mean Fluorescence Intensity (MFI).

Apoptosis

Phosphatidylserine, which is normally only present on the inner leaflet of the plasma membrane of cells, becomes expressed on the outer leaflet of the membrane of apoptotic cells.[27] Annexin V is a protein that can bind to phospholipids, and especially to phosphatidylserine, in a calcium-dependent fashion,[28] and is therefore used as a marker for apoptosis of cells. Annexin V was prepared, purified and labelled with FITC as described before.[13] Annexin V binding to neutrophils was measured by direct immunofluorescence as described earlier.[13] The binding of Annexin V to PMN was expressed as percentage positive cells.

Chemotaxis

The Boyden chamber (48-well chemotaxis chamber, Neuro Probe Inc., Cabin John, MD) was used for measurement of PMN chemotaxis. The Boyden chamber contained an 8.0-µm migration filter on top of an 0.45-µm arresting filter. Chemotactic stimuli, added to the lower compartments of the Boyden chamber, were either FMLP (10^{-8} M), C5a (10^{-8} M), PAF (10^{-7} M), or IL-8 (10^{-8} M). Incubation medium for cell suspensions, as described earlier, served as control. Indicated populations of PMN were suspended in incubation medium for cell suspensions, supplemented with heparin (10 IU/ml). 10^5 PMN were added to the upper compartment of the Boyden chamber and incubated for 1.5 h. Thereafter, the 8.0-µm Boyden chamber filters were stained, dehydrated with xylol and the number of PMN on the bottom of this filter were counted with a light-microscope at 400 × magnification. Chemotaxis was defined as the mean of the number of migrated PMN counted in five microscopic fields.

RESULTS

Eight CAPD patients enrolled in the study. The diagnosis of peritonitis was made by the observation of a cloudy bag. The effluent neutrophils examined

in this study were from this first cloudy bag. The diagnosis of peritonitis was confirmed in all patients by positive cultures from the effluent. *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Difteroïds* each in two patients caused the episodes. *Candida albicans* and *Micrococcaceae* were both in one patient the micro-organism cultured from the effluent.

Surface Ag expression

L-selectin expression (detected with Leu-8) was decreased in the patients' effluent PMN compared to the PMN isolated from the peripheral blood of patients. There was no significant difference in L-selectin expression between the patients' blood PMN and control blood PMN (Table 1). The expression of all FcR receptors- FcRI (CD64), FcRII (CD32) and FcRIII (CD16), on the PMN of the patients was equal to control PMN (Table 1). The expression of the β_2 integrin adhesion proteins CD11b and CD11c on the patients' effluent PMN was significantly increased compared to the patients' peripheral PMN. Interestingly, this increase was less pronounced and not significant for their common β-chain CD18 (Table 1).

Table 1. Surface antigens present on peripheral blood, and effluent PMN during peritonitis in CAPD patients

mAb	controls blood PMN	CAPD patients blood PMN	CAPD patients effluent PMN
Ctrl. 1	31 ± 3.5	27 ± 4.2	35 ± 3.5
Ctrl. 2	36 ± 5.5	28 ± 2.8	28 ± 3.6
CD10	146 ± 14.4	103 ± 12.0	135 ± 15.1 *3
CD11a	229 ± 15.1	174 ± 16.7 *1	193 ± 18.2
CD11b	384 ± 85.8	552 ± 66.7	1054 ± 172.8 *3
CD11c	119 ± 13.0	102 ± 13.0 *1	144 ± 13.9 **3
CD16	1407 ± 410.3	1476 ± 473.0	1357 ± 354.9
CD18	189 ± 36.3	182 ± 31.5	236 ± 49.6
CD32	279 ± 32.4	260 ± 22.8	281 ± 25.4
CD35	116 ± 14.9	120 ± 13.0	157 ± 26.5
CD45	260 ± 31.2	250 ± 27.6	351 ± 50.9 *3
CD63	40 ± 4.8	32 ± 3.0	38 ± 4.1
CD64	40 ± 6.7	48 ± 6.1	44 ± 5.5
CD66b	105 ± 17.6	165 ± 32.2 *2	277 ± 51.5 *3
Leu-8	535 ± 36.2	475 ± 21.9	136 ± 26.9 **4

Table 1. Results are expressed as the mean ± SEM of eight experiments. Statistics were performed with the paired *t*-test. * $p \leq 0.05$, ** $p \leq 0.01$. [1]Expression of patients' blood PMN was significantly lower compared to control blood PMN. [2]Expression of patients' blood PMN was significantly higher compared to control blood PMN. [3]Expression of effluent PMN was significantly higher compared to patients' blood PMN. [4]Expression of effluent PMN was significantly lower compared to patients' blood PMN.

After resolution of the peritonitis, CD66b expression on the patients' peripheral PMN was not different from control blood PMN (data not shown); CD11a and CD11c expression was again lower on peripheral blood PMN of CAPD patients *versus* control blood PMN, but these latter differences were not significant (data not shown). Remarkably, the expression of CD10 on control PMN was higher compared to that on peripheral blood PMN from the CAPD patients and this phenomenon stayed present after the recovery of peritonitis.

Apoptosis

Annexin V binding to the PMN was observed in only few PMN of both patients and healthy volunteers. Essentially $\leq$ 1% of the cells in each of the 3 different PMN populations were apoptotic (Table 2).

Table 2. Annexin V binding to control PMN, peripheral blood PMN, and effluent PMN during the onset of clinical symptoms of CAPD-associated peritonitis.

	Annexin V positive cells (%)
control blood PMN	1.1 ± 0.4
patients blood PMN	0.4 ± 0.1
patients effluent PMN	1.0 ± 0.5

Results are expressed as the percentage ± SEM of positive cells.

Chemotaxis

Neutrophil migration towards Hepes medium was minimal for all types of PMN. The migration of the patients' blood PMN towards PAF or IL-8 was slightly reduced compared to that of control blood PMN. A further reduced chemotaxis was observed in case of patients' effluent PMN. However this was not significant (paired *t*-test; Figure 1). After resolving of the peritonitis, the differences in chemotactic response of the control blood PMN *versus* that of the patients' blood PMN to IL-8 and PAF were less pronounced (data not shown). During peritonitis, all 3 populations of PMN showed no difference in chemotaxis towards FMLP or C5a. C5a was the most potent chemoattractant for neutrophils in this set-up (Figure 1).

DISCUSSION

Neutrophils are the predominant cells found in the effluent of patients at the beginning of CAPD-associated peritonitis. These PMN are essential as the first-line defence against microbial infections.[29] However, they can also mediate pathologic (side) effects such as tissue destruction at the site of

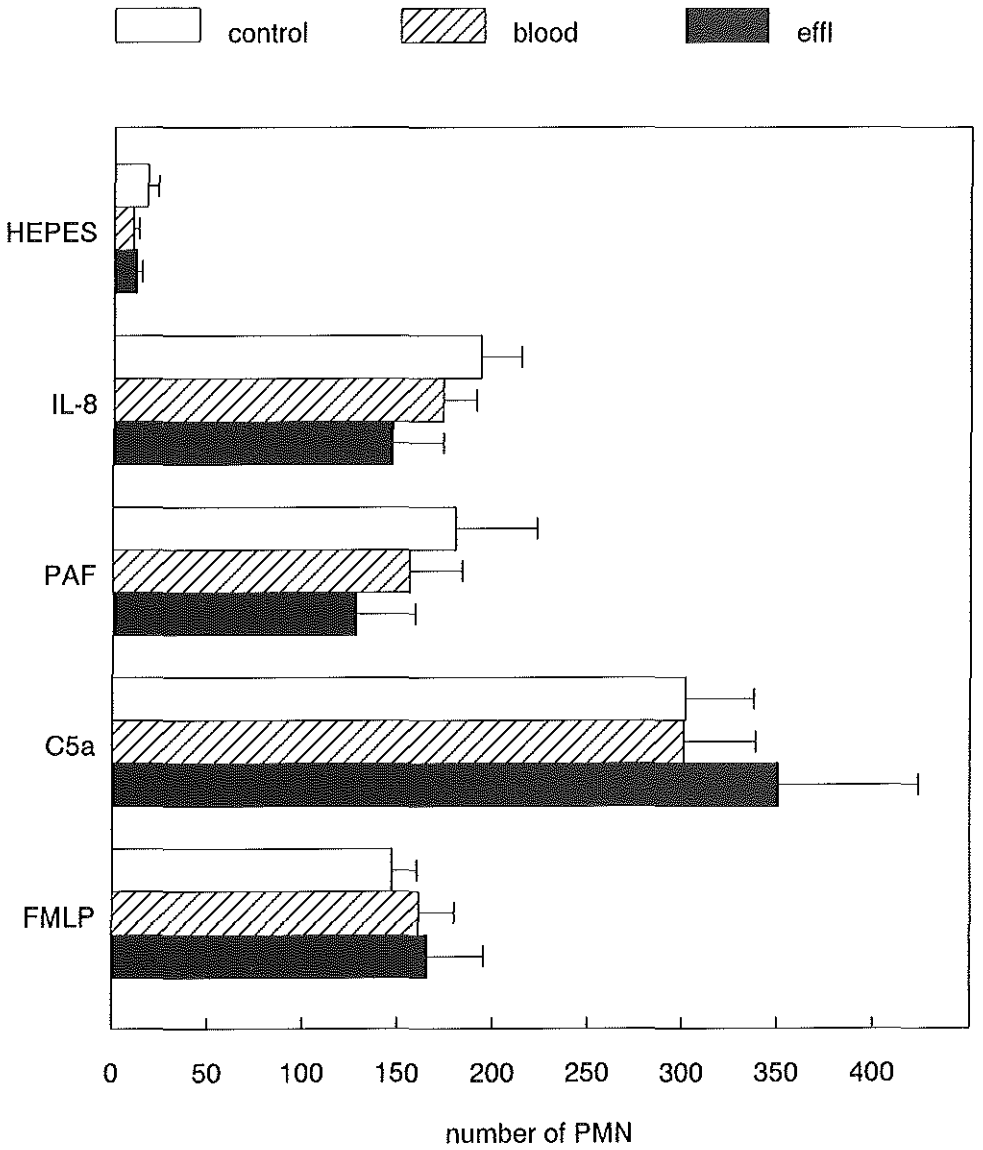

Figure 1. Chemotaxis of neutrophils isolated from peripheral blood and effluent of CAPD patients at the time of peritonitis. Control neutrophils were isolated from the peripheral blood of healthy donors. Chemotaxis was measured towards IL-8, PAF, C5a or FMLP in a Boyden chamber. Incubation medium for cell suspension (HEPES) served as control attractant. Results are expressed as the mean ± SEM of eight separate experiments.

inflammation.[30] The function of the PMN during inflammation depends on two mechanisms: the NADPH oxidase system, which provides toxic oxygen metabolites, and the proteolytic capacity of enzymes stored in the cytoplasmic granules of the PMN.[31, 32]

The influx of neutrophils into inflammatory tissue is a well-coordinated process that involves locally produced cytokines and requires active participation of adhesion receptors. The adherence of neutrophils to other cells is dependent on the family of β_2 integrin proteins (CD11/CD18), of which CR3 (CD11b/CD18) is the most important leukocyte adhesion molecule involved in neutrophil adherence.[33, 34]

In our study, the expression of CD11b as well as CD11c on PMN in effluent was significantly increased compared to those on peripheral blood PMN collected from the same patients at the same time. The expression of the common β-chain CD18 was also higher on dialysate PMN *versus* blood PMN, but this difference was not statistically significant. The intracellular storage site for CD11b in neutrophils are the specific granules,[35] and the secretory vesicles.[36] Activation of granulocytes in vitro rapidly increases the expression of CD11b receptor on the outer membrane[36, 37] Upregulation of CD11b and CD11c together with CD45 (membrane-associated tyrosine phosphatase) and CD66b (a CEA-like antigen), present in the specific granules and secretory granules in PMN,[12] occurred during extravasation into the abdominal cavity during peritonitis. The upregulation described concurs with previous findings by Jones et al. (with the exception of CD66b; which they did not test) who compared circulating neutrophils with PMN isolated from joint fluids in patients with rheumatoid arthritis.[38]

Neutral endopeptidase (CD10), stored exclusively in secretory vesicles, can be considered as an 'early activation antigen.'[12] This concurs with our finding that CD10 expression was increased in effluent *versus* circulating PMN.

In contrast to the upregulation of the previous membrane antigens, L-selectin was downregulated on effluent neutrophils. Shedding of L-selectin from the granulocyte membrane upon activation or migration into tissues is a well-known phenomenon.[39-41] CD63, a specific marker for azurophilic granule fusion with the plasma membrane of neutrophils,[12] was not upregulated on dialysate PMN, indicating that during the extravasation of neutrophils, where PMN migrate across endothelium and mesothelium to reach the peritoneal cavity, no trigger for release of azurophilic granules had occurred. This finding concurs with data obtained from in vitro and in vivo experiments.[42, 43] Although upregulation of adhesion proteins on granulocytes and release of proteins from specific granules coincide with neutrophil migration across endothelium, these changes in 'activation state' of the neutrophil are not required for efficient endothelial transmigration in vitro.[42]

The expression of surface Fc receptors was not different between the patients' peripheral and effluent PMN. The expression levels of FcRI remained equal to control blood mAb, which would suggest that these neutrophils in CAPD peritonitis had not been exposed to interferon gamma (IFN-γ).[44]

Decreased responsiveness (desensitization) to one or more chemotactic factors occurs after exposure of neutrophils to a high concentration of a chemotactic factor. Such exposure is usually followed by a decrease in specific receptors on the PMN's membrane.[45] Kuijpers et al. have shown that neutrophil migration across activated endothelium *in vitro* is guided by endothelium derived PAF and IL-8.[2] Our previous studies have shown that activated mesothelial monolayers are able to create, through polarized secretion of IL-8, a chemotactic gradient of IL-8 directed towards the abdominal cavity.[4] It is likely that the same regulatory mechanisms occur in vivo, because the present study provides possible evidence for preferential deactivation in chemotaxis of CAPD effluent PMN towards IL-8 and PAF when compared to the peripheral blood PMN of the same patients at the same time (Figure 1). However, this effect did not reach statistical significance (paired *t*-test). We did not observe a preferential deactivation of effluent PMN towards FMLP or C5a.

Apoptosis, also called programmed cell death,[46] was not detected in the PMN in this study. The neutrophils of patients with CAPD-associated peritonitis were isolated and investigated within 3 hrs after the first cloudy effluent was observed, which is often the first clinical symptom of this complication. Apoptosis in neutrophils is, thus, not apparent during the early phase of bacterial peritonitis in CAPD.

From the data presented in our study it can be concluded that during the initial stage of CAPD-associated peritonitis effluent neutrophils have actively changed their marker profile and thus 'activation state' of PMN. Also, a modest modulation of chemotactic responsiveness of the dialysate neutrophilic granulocytes is observed towards IL-8 and PAF.

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7

LIMITED INFLUENCE OF THE MESOTHELIUM ON THE INFLUX OF MONOCYTES INTO THE PERITONEAL CAVITY

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ABSTRACT

We have investigated the role of human mesothelium in an *in vitro* model of peritonitis on the monocyte adherence to and migration across monolayers of peritoneal mesothelial cells. Monocytes adhere avidly to non-activated mesothelial cells monolayers; however, migration in this situation was minimal. Prestimulation of the monolayers with Il-1β did not alter these results. Anti-CD18 and anti-VLA-4 mAbs used in combination had an additive inhibitory effect on monocyte adherence to resting or IL-1β-pretreated mesothelial cells. MCP-1 and TGF-β are secreted by mesothelial cells. Both have a modest role in mesothelium-induced monocyte chemotaxis: mAbs against these cytokines had an additive inhibitory effect on the chemotaxis induced by supernatant from 24-h prestimulated mesothelial cells. Our results indicate that the mesothelium itself has a limited role in the influx of monocytes into the peritoneal cavity during the onset of peritonitis.

INTRODUCTION

As an initial event in acute bacterial peritonitis, a massive influx of neutrophils is observed into the peritoneal fluid, followed within 24 hours by the migration of large numbers of monocytes.[1] Previous in vitro studies have shown that activated mesothelium stimulates the neutrophil influx by inducing neutrophil adherence[2] and by secreting cytokines.[3-6] The latter process induces a rapid and strong migration of neutrophils across mesothelial cell monolayers.[6] Monocyte migration across *endothelial* monolayers seems to occur much like the neutrophil transmigration, and requires also the interaction of specific adhesion surface molecules and locally produced cytokines.[3,7,8] The present study was designed to investigate the qualitative role of mesothelial adhesion receptors and mesothelial-derived cytokines in monocyte adherence to and migration across cultured monolayers of human mesothelial cells.

MATERIALS AND METHODS

Monoclonal antibodies (mAb)

The following mAb were used. mAb against E-Selectin (ENA2 [IgG_1]),[9] VCAM-1 (4B9 [IgG_1]),[10] and ICAM-1 (RR1/1 [IgG_1]),[11] were used for immunofluorescence assays. The influence of the following mAbs was studied in the adherence and migration assay: CLB-LFA-1/1 (IgG_1)[12] against the common β chain CD18 of the CD11/CD18 family of β_2 integrins and mAb HP1/3 (IgG_3) against the α chain of VLA-4 (CD49d).[13] mAb against transforming growth factor-β (TGF-β; R&D systems, Minneapolis, MN) and Ab against monocyte chemotactic protein-1 (MCP-1; polyclonal rabbit anti-human MCAF; Genzyme Corporation, Cambridge, MA) were used to study the influence of mesothelium-derived cytokines in the chemotaxis of monocytes in a modified Boyden chamber assay.

Mesothelial cell culture

Mesothelial cells (MC) were isolated from human omentum according to techniques modified from Nicholson et al.[14] and Wu et al.,[15] as previously described.[16] In brief, small pieces of omentum were removed early in the operative procedure from patients undergoing abdominal surgery for non-infectious conditions. All patients gave informed consent. The omentum was transferred to fluid containing 0.05% (w/v) trypsin-0.02% (w/v) EDTA (Gibco, Life Technologies, Paisley, UK). After 15 min the detached MC were pelleted by centrifugation at 1200 rpm for 5 min and resuspended in supplemented M-199 medium (Gibco). MC were grown until confluence in a 37°C, fully humidified, 5% CO_2 cabinet in polystyrene culture flasks (75 cm^2; Costar, Cambridge, MA) precoated with fibronectin. The identity of MC was demonstrated by the absence of von Willebrand factor staining[17] and the presence of intracellular cytokeratins by immunofluorescence with monoclonal antibodies[18] (Dakopatts, Glosstrup, Denmark).

Determination of surface antigen expression

The expression of the adhesion molecules E-Selectin, ICAM-1 and VCAM-1 by mesothelial cells upon activation was measured by FACS analysis. MC were subcultured to confluent monolayers in 6-well culture dishes (Costar). The MC were activated by incubation with human recombinant interleukin-1β (IL-1β) (25 U/ml; Genzyme Corporation) for various periods. A control well with MC was not incubated with IL-1β. After activation, the MC were non-enzymatically detached by incubation with PBS/EDTA (2 mM) for 15 min at 37°C. MC in suspension were washed twice in ice-cold PBS and were subsequently incubated with the primary mAbs for 30 min at 4°C. Thereafter, the cells were washed twice in ice-cold PBS, containing 0.5% (v/v) bovine serum albumin (BSA), and the procedure was repeated with FITC-labeled goat-anti-mouse-Ig for another 30 min at 4°C. After two washes, mAb binding was quantified for 10,000 cells with a FACScan (Becton Dickinson, Mountain View, CA) and was expressed as mean fluorescence intensity (MFI).

Isolation of monocytes

Blood was obtained from healthy volunteers and anticoagulated with 0.4% (w/v) trisodium citrate (pH 7.4). Monocytes were purified from buffy coats as described.[19] In brief, blood cells were centrifuged over isotonic Percoll (specific gravity at room temperature, 1.077/cm^3). The interphase, containing the mononuclear cells, was taken for isolation of the monocytes by countercurrent centrifugal elutriation.[19] Purity of the monocytes was ≥95%, and viability remained ≥95%, as determined by lactate dehydrogenase (LDH) release.[20]

Labeling of monocytes

Freshly purified monocytes were radiolabeled with ^{51}Cr according to Gallin et al.[21] with minor modifications. Briefly, monocytes were incubated at 4°C for 15 min with 0.5 μCi of ^{51}Cr per 10^6 cells (sodium chromate, 200 to 500 Ci/g; New England Nuclear, Boston, MA) in 50% (v/v) M-199 (Gibco Life Technologies, Paisley, UK), 50% (v/v) RPMI-1640 (Gibco), supplemented with 0.1% (v/v) HSA. The cells were subsequently washed and resuspended in incubation medium for cell suspensions, containing 20 mM Hepes, 132 mM NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 6 mM KCl, 1.2 mM KH_2PO_4, 5.5 mM glucose and 0.5% (w/v) HSA, pH 7.4 at 4°C. Incubation of the monocytes took place for 30 min, during which the temperature of the suspension was gradually increased to room temperature. Subsequently, the monocytes were washed twice and resuspended in 50% (v/v) M-199, 50% (v/v) RPMI-1640, 0.5% (v/v) HSA at 37°C . Aggregated cells were removed by cotton-wool filtration, and the remaining cells were resuspended in fresh incubation medium at a concentration of 10^6 cells/ml. Viability after labeling remained ≥95%.

Migration assay

Inverted filters (with MC 'hanging' on the filters) were used to study migration in the physiologically relevant direction (submesothelial tissue to peritoneal cavity), thus permitting the monocytes to adhere

to the basolateral side of the MC monolayer. MC were subcultured to confluent monolayers on inverted polycarbonate membranes (8.0 µm pore size, 24.5 mm diam) of Transwell cell culture chamber inserts (Costar), according to Parkos et al.[22] with minor modifications, as previously described.[6] MC monolayers reached confluence in five days as determined by phase-contrast microscopy, microscopy of May-Grünwald/Giemsa stained filters and by electrical resistance measurements.[6] In some experiments the monolayers were preincubated with IL-1β (25 U/ml) added to the lower compartment during 6 hours prior to adding the monocytes. IL-1β had no effect on the morphology of the cells, nor did it influence the electrical resistance of the monolayers.[6] When indicated, formyl-leucyl-methionyl-phenylalanine (FMLP; Sigma Co., St. Louis, MO) was added to the lower compartment in a final concentration of 10^{-6} M. In all experiments the upper compartment of the cell culture chamber inserts was washed twice with 50% (v/v) M-199, 50% (v/v) RPMI-1640, 0.5% (v/v) HSA prewarmed to 37°C before adding the monocytes. The fluid in the lower compartment was not replaced. ^{51}Cr-labeled monocytes (10^6 cells/ml) prewarmed to 37°C were added to the upper compartments. The chamber plates were incubated at 37°C in a 5% CO_2 incubator for 60 min. After incubation, the fluid of each compartment was collected. The amount of radioactivity present in the fluids as well as that of the filter, removed from its cylindric container, were determined in a gammacounter. Recovery was always ≥92%. The radioactivity measured in the filter was taken to represent monocyte adhesion. The extent of migration was calculated from the radioactivity found in the fluid of the lower compartment. The results were expressed as percentages of the total radioactivity, i.e. monocytes, added to the chambers.

Measurement of chemotaxis

The Boyden chamber (48-well chemotaxis chamber; Neuro Probe Inc., Cabin John, MD) was used for measurement of monocyte chemotaxis. The Boyden chamber contained an 8.0-µm migration filter on top of an 0.45-µm stopping filter. Chemotactic stimulus, added to the lower compartment of the Boyden chamber, consisted of mesothelial cell supernatant. These supernatants were obtained from non-stimulated or rIL-1β-prestimulated mesothelial cells monolayers, cultured on 6-well plates (Costar) during 6 or 24 hours. Culture medium (supplemented M-199) and FMLP (10^{-6}M; Sigma Co.) diluted in culture medium were used as negative and positive controls, respectively. When indicated, mAb against MCP-1 or Ab against TGF-β was added to the supernatant as well as to the monocytes 10 min prior to adding the monocytes. Monocytes were suspended in incubation medium for cell suspensions, as described earlier, supplemented with heparin (10 IU/ml). 10^5 monocytes were added to the upper compartment of the Boyden chamber and incubated for 2.5 hours. Thereafter, the 8.0-µm Boyden chamber filters were removed, fixed in butanol/ethanol (20/80%, v/v) for 10 min and stained with Weigert solution (1% v/v), i.e. hematoxylin in ethanol mixed with a 70 mM acidic $FeCl_3$ solution at a 1:1 ratio. The filters were dehydrated with ethanol, made transparent with xylol and fixed upside down. The chemotactic response of the monocytes was quantified with an image analyzer (Quantimet 570C, Leica Cambridge Ltd.,Cambridge, UK) using Quantimet 570 Control Software (QUIC, version 2.02) and a custom made software program written in Q Basic.[23] An automated microscope was used to step through the filters at a given number of levels in the Z direction with a certain Z level interval. Cells were counted at each of these levels. Chemotaxis was expressed as the mean monocyte migration distance into the filter in µm from the surface of the filter.

RESULTS

Adhesion receptors

ICAM-1 and VCAM-1 are present on the surface of non-activated MC in significant numbers (Table 1, Figure 1). After activation of the MC with IL-1β, the expression of both adhesion proteins increased with a maximum expression for both proteins on the cell membrane after 6 to 8 h. Thereafter, the expression

of ICAM-1 and VCAM-1 gradually decreased, but remained above background after 24 hrs and even after 48 hrs (Table 1). Activation of the MC with TNF-α instead of IL-1β showed similar increases in ICAM-1 and VCAM-1 expression, with the same kinetic profile (data not shown). E-Selectin was not detectable on either resting or activated MC: binding of anti-E-Selectin mAb never exceeded levels with control mAbs (Table 1).

Table 1. Surface antigen expression of adhesion molecules on mesothelial cells at various times after IL-1β addition.

Time after IL-1β induction (hrs)	E-Selectin	ICAM-1	VCAM-1
0	9 ± 6	2156 ± 465	584 ± 121
2	11 ± 8	2620 ± 559	828 ± 163 *
4	12 ± 7	3056 ± 681 *	821 ± 183 *
6	11 ± 9	3416 ± 492 **	1029 ± 237 *
8	29 ± 16	3444 ± 638 **	1082 ± 306
16	17 ± 8	2996 ± 333 **	874 ± 234
24	20 ± 13	2950 ± 508 **	722 ± 149 *
48	11 ± 7	2414 ± 485 *	787 ± 196

Table 1. Results are expressed as mean fluorescence intensity (MFI) ± SEM of 5 separate experiments. * $p \leq 0.05$; ** $p \leq 0.01$, significant increase in membrane expression compared to non-stimulated mesothelial cells (paired *t*-test).

Monocyte adherence and migration

Monocytes adhered avidly to non-activated MC monolayers. However, migration in this situation was minimal (Figure 2). Activation of the MC monolayers with IL-1β for 6 h showed little modulating effect: the adherence of monocytes to MC monolayers increased marginally, but still only very small numbers of monocytes transmigrated across the MC monolayers (Figure 2).

CD18 mAb CLB LFA1/1 had little effect on monocyte adherence to cytokine-activated MC monolayers. The same effect was seen after incubation of the monocytes with anti-VLA-4 mAb HP1/3. However, CD18 and anti-VLA-4 mAbs used in combination had a synergistic inhibitory effect on monocyte adherence to IL-1β-pretreated cells (85% inhibition, $p \leq 0.01$) (Figure 2). The same inhibitory effect of this combination of mAbs was observed when monocyte adherence to non-stimulated MC monolayers was measured (data not shown). FMLP added to the lower compartment of the Transwell system induced a small but significant increase in migration of the monocytes across non-stimulated MC monolayers ($p \leq 0.05$) (Figure 2).

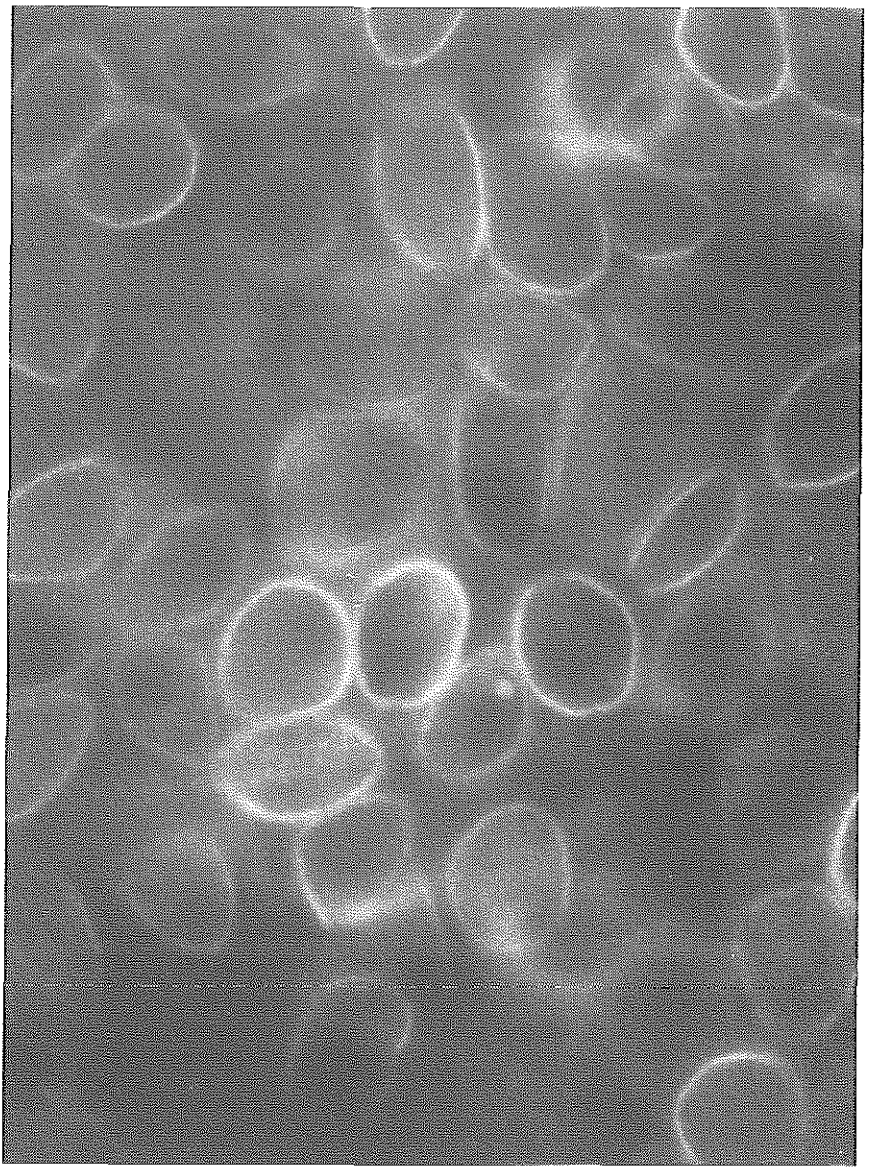

Figure 1. Surface expression of ICAM-1 on cultured non-activated mesothelial cells.

Chemotaxis of mesothelial supernatant

The mean migration distance of monocytes towards fresh culture medium in Boyden chambers was minimal (Figure 3). In contrast, MC supernatant induced a significantly increased monocyte chemotaxis, comparable to levels induced by

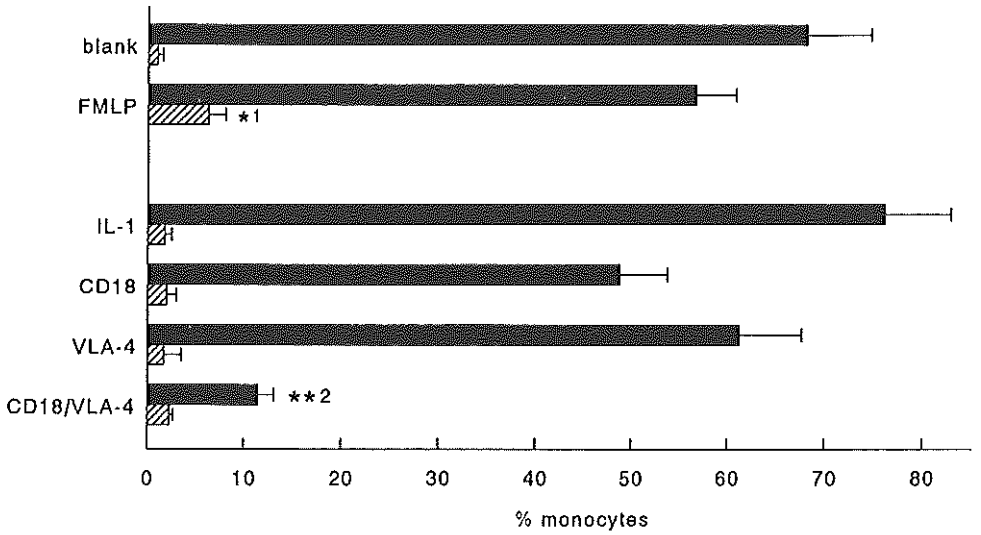

Figure 2. Monocyte adherence to and migration across inverted monolayers of MC. Mesothelial cells were cultured on inverted 8.0 µm filters, and monocyte adherence and migration were measured 60 min after addition of the monocytes. Blank represents adherence to and migration across resting MC. Effects of mAb against CD18 and VLA-4 on IL-1β induced monocyte adhesion to and migration across inverted MC monolayers are shown. Black bars represent monocyte adherence to, and shaded bars represent monocyte migration across MC monolayers. Results are expressed as the mean ± SEM of three separate experiments. [1]Monocyte migration was significantly increased compared to migration across non-activated MC monolayers. [2]Adhesion of monocytes to inverted MC monolayers was significant decreased compared to adhesion to IL-1β-activated MC monolayers (paired *t*-test). * $p \leq 0.05$, ** $p \leq 0.01$.

FMLP ($p \leq 0.05$) (Figure 3). Changes in the 'activity-state' of the MC played a minor role, because activation of the MC monolayers with IL-1β, even for 24 hrs, did not have an additive stimulating effect on the chemotaxis of the monocytes towards the culture medium (Figure 3).

Anti-MCP-1 Ab had an inhibitory effect on the chemotaxis of mesothelial cell-derived supernatant, but this effect was only significant in supernatant of 24 hrs IL-1β prestimulated MC monolayers ($p \leq 0.05$) (Figure 3). Anti-TGF-β Ab had a minor effect on supernatant from non-stimulated MC cells, but a significant reducing effect on supernatant obtained from 24 hrs IL-1β activated MC monolayers ($p \leq 0.05$) (Figure 3). Furthermore under these circumstances, anti-MCP-1 and anti-TGF-β used in combination had a synergistic inhibitory effect on the monocyte chemotaxis ($p \leq 0.01$) (Figure 3), to levels comparable to the migration distance measured towards fresh culture medium. Chemotaxis induced by FMLP was not influenced by Abs against MCP-1 or TGF-β (data not shown).

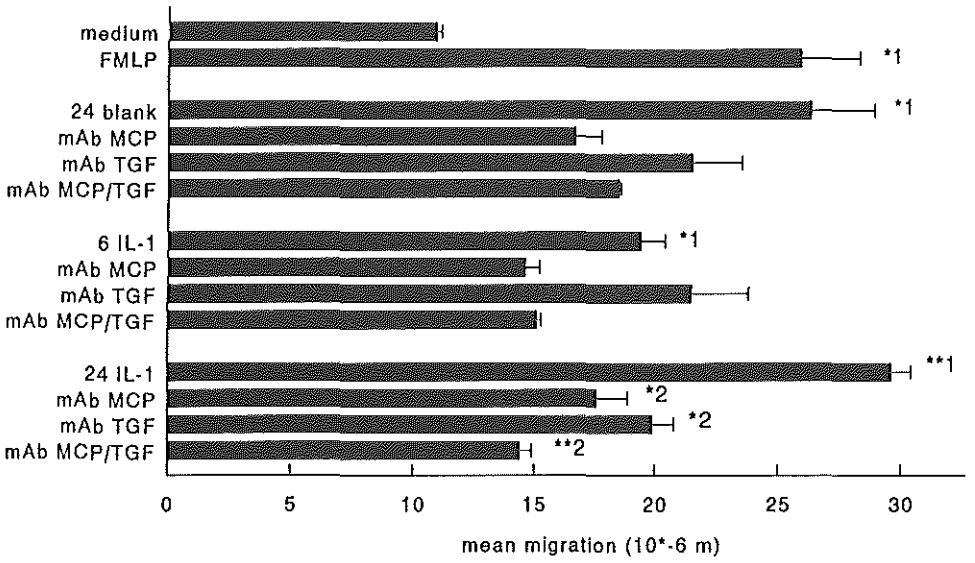

Figure 3. Chemotaxis of monocytes in Boyden chambers (2.5 hrs) induced by supernatant from non-activated, and from 6 h or 24 hrs IL-1β pretreated mesothelial cell monolayers. The monocyte chemotaxis measured towards culture medium and towards FMLP were used as negative and positive controls, respectively. The chemotactic response was quantified with an image analyzer, as described in the Material and Methods section. Cells were counted at small levels spread 10 µm apart throughout the whole filter (150 µm). Results are expressed as the mean migration distance ± SEM of three separate experiments. [1] Mean migration distance was significantly higher than the distance towards (culture) medium. [2] Mean migration distance was significantly lower than the distance induced by supernatant from MC monolayers prestimulated during 24 h with IL-1β (paired *t*-test). * $p \leq 0.05$, ** $p \leq 0.01$.

DISCUSSION

Adherence of monocytes to and migration across activated endothelial cell monolayers is a process that involves adhesion receptors on both types of cells and depends on locally produced cytokines.[3,7,8] In peritonitis, monocytes have to transmigrate not only across an endothelial cell barrier but also across a mesothelial cell monolayer before they can enter the abdominal cavity. Previous studies on the role of mesothelium in this process have been performed by Gerwin et al.[5] who described the presence of mRNA for TGF-β in MC, a chemoattractant for PMN and monocytes. Jonić et al.[3] detected mRNA expression for MCP-1 in activated MC and also described the presence of ICAM-1 and VCAM-1 on MC monolayers. In our study we describe the qualitative role of adhesion receptors in monocyte adherence to and the lack of importance of some MC-derived cytokines in monocyte migration across MC monolayers.

Adherence of monocytes to MC is -like adherence of monocytes to endothelial cells[7]- a combined CD18/ICAM-1 and VLA-4/VCAM-1 dependent process. E-Selectin is not expressed on the membrane of MC monolayers and, therefore, plays no role in monocyte adherence. No alteration in the level of adhesion was found even when monocyte adherence was measured 6 hrs after activation with IL-1β, at the time of maximal expression of both VCAM-1 and ICAM-1. In a previous study[2] we reported that an increase in ICAM-1 expression on the MC membrane itself was insufficient for neutrophil adherence. 'Slow travelling' of monocytes in our system with Transwell filters resulted in minimal monocyte transmigration across activated MC monolayers. In contrast, PMN are able to transmigrate rapidly under the same conditions (23% in 30 min);[2] however, PMN were not able to adhere to and transmigrate across non-activated MC monolayers as do monocytes.[2] Thus, activation of the MC monolayer modulates to a large extent the PMN adherence to and migration across these cell layers, but has little influence on monocytes adherence and migration.

Boyden chamber experiments with supernatant of 24 hrs IL-1β prestimulated MC showed that its chemotactic activity was inhibited by adding Abs against MCP-1 and TGF-β. This inhibition supports a role for these monocyte chemoattractants in monocyte recruitment towards the abdominal cavity during peritonitis. However, significant monocyte chemotaxis was also measured in the Boyden chamber towards supernatant from non-activated MC. This finding concurs with the *in vivo* situation that during continuous ambulatory peritoneal dialysis (CAPD) a population of resident macrophages of monocytic origin is found in the peritoneal effluent even in the absence of inflammation, i.e. without the presence of neutrophils.[24,25] In the event of peritonitis, circulating monocytes from outside the peritoneal cavity will be attracted to the site of inflammation by chemotaxis,[26,27] preceded by a massive influx of neutrophilic granulocytes.[8,28]

From the data presented in this study it is concluded that, in contrast to PMN migration, the mesothelium itself has little modulating effect on the process of transmesothelial cell migration of monocytes which takes place in the inflammatory response during bacterial peritonitis.

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8

CA 125 SECRETION BY PERITONEAL MESOTHELIAL CELLS

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ABSTRACT

An in vitro model of peritonitis was used to investigate the secretion of the tumormarker CA 125 by cultured human mesothelial cells. The apical versus basolateral secretion of CA 125 was measured and it was determined if enhanced secretion of CA 125 could be observed by activating the mesothelial monolayers with different cytokines. Mesothelial cells were isolated from human omentum and cultured to confluent monolayers on perforated polycarbonate membranes (pore size 0.4 μm). The mesothelial monolayers were activated and the apical and basolateral secretion of CA 125 compared. To investigate the influence of cytokines, mesothelial cells were cultured and activated with recombinant interleukin-1β (rIL-1β), tumor necrosis factor-α (TNF-α) or lipopolysaccharide from *Escherichia coli*. The secretion of CA 125 was tested using a microparticle enzyme immunoassay. Mesothelial monolayers secreted CA 125 in a polarized manner with preference for the apical side. Apical polarization occured irrespective of the side of the inducing stimulus ($p \leq 0.05$). Non-activated cultured mesothelial monolayers secreted significant quantities of CA 125, indicating constitutive production of this protein. However, CA 125 production was significantly enhanced if mesothelial cells were incubated with rIL-1β ($p \leq 0.05$), TNF-α ($p \leq 0.05$), and *E. coli* LPS ($p \leq 0.01$). Human mesothelial monolayers secrete CA 125 preferentially from their apical surfaces. The secretion of CA 125 can be enhanced by the inflammatory cytokines Il-1β, TNF-α, and by *E. coli* LPS.

INTRODUCTION

Cancer Antigen 125 (CA 125) is a 220,000 molecular weight glycoprotein defined by a murine monoclonal antibody OC 125, which was raised against the serous ovarian carcinoma cell line OVCA 433.[1] Immunohistochemical studies have shown that CA 125 is expressed by coelomic epithelium during embryonic development and by most ovarian epithelial tumors. CA 125 was detected in serum samples of about 80% of patients with ovarian cancer.[2] Subsequently, CA 125 was a clinically useful tumormarker in the follow-up of patients treated for ovarian adenocarcinomas.[3 4]

However, CA 125 proved not to be a tumor specific antigen, as its presence was shown in the normal epithelium of the female genital tract; in gastric and colonic mucosal cells; and at the luminal surface of mesothelium lining the peritoneum, pleura and pericardium.[5]

High concentrations of CA 125 have been found in the sera of patients after abdominal surgery and in patients during episodes of bacterial peritonitis. These findings suggest that mesothelial cells are active in CA 125 synthesis en secretion.[6-8]

We investigated the CA 125 secretion of human mesothelial cells in an in vitro model of peritonitis. The study focused on the secretion pattern of CA 125 by a mesothelial cell monolayer and the enhancement of mesothelial cell CA 125 secretion by several inflammatory stimuli.

MATERIALS AND METHODS

MC were isolated from human omentum, according to techniques modified from Nicolson and Wu.[9,10] Small pieces of omentum were removed early in the operative procedure from patients undergoing abdominal surgery for non-infectious conditions. All patients gave informed consent. After removal, the pieces of omentum were placed in medium M199 (Gibco, Life Technologies, Paisley, Scotland) at 37°C. Within two hours the pieces of omentum were transferred to PBS containing 0.05% trypsin-0,02% EDTA (Gibco). After 15 min incubation at 37°C under gentle shaking, the detached mesothelial cells were pelleted by centrifugation at 1200 rpm for 5 minutes. The pelleted cells (mesothelial cells with other cells, predominantly erythrocytes) were resuspended in M199 supplemented with fetal bovine serum (FBS; Gibco), gentamycin (10 µg/ml; Merck, Darmstadt, Germany), vancomycin (25 µg/ml; Gibco) and glutamine (2 mM). The cells were grown until confluence in a 37°C, fully humidified, 5% CO_2 cabinet in polystyrene culture flasks (75 cm^2, Costar, Cambridge, Massachusetts, USA) precoated with fibronectin. Mesothelial cells were identified by immunofluorescent staining using mouse monoclonal antibodies against the cytokeratins 6 and 18.[11]

To investigate the apical and basolateral secretion of CA-125, mesothelial cells were subcultured to confluent monolayers on polycarbonate membranes (0.4 µm pore size, 24.5 mm diameter) of Transwell cell culture chamber inserts (Costar). The filters were precoated with fibronectin before mesothelial cells were added. MC monolayers reached confluence in 5 days as determined by May-Grünwald/Giemsa staining. Pretreatment of the monolayer with human recombinant interleukin-1β (rIl-1β; 25 U/ml; Genzyme, Corporation, Boston, Massachusetts, USA) did not influence the microscopic morphology of the confluent monolayer. In all experiments culture medium without antibiotics was used. Medium was refreshed prior to adding rIl-1β to either the upper or lower compartment of the Transwell-system. At nought and six hours, samples from both compartments were taken and stored at -70°C until tested for CA 125.

To find out whether other stimuli besides rIl-1β were able to induce the mesothelial cells to secrete CA 125, these were subcultured to confluent monolayers in six well culture dishes (Costar) The wells were incubated with tumor necrosis factor-α (TNF-α; 100 U/ml; Genzyme Corporation), lipopolysaccharide from Escherichia coli (LPS; 1 µg/ml; Sigma Chemical Co., St. Loius, Missouri, USA) or rIl-1β (25 U/ml). A well without a stimulus was used as control. Six hours after incubation, samples of the supernatant fluids were taken and stored at -70°C untill tested for CA 125.

A microparticle enzyme immunoassay (MEIA) was used (IMx CA 125, Abbott Laboratories, AbbottPark, Illinois, USA) for the quantative measurement of secreted human CA 125 by the mesothelial cell monolayers.

RESULTS

Activated MC monolayers noticeably show a polarity in secretion of CA 125 in favour of the apical side of the monolayer. Whether the stimulus is introduced to the apical or basolateral side of the mesothelial cell monolayer seems to be of no importance, as both modes of stimulation lead to preferential secretion of CA 125 towards the apical side of the monolayer (Figure 1).

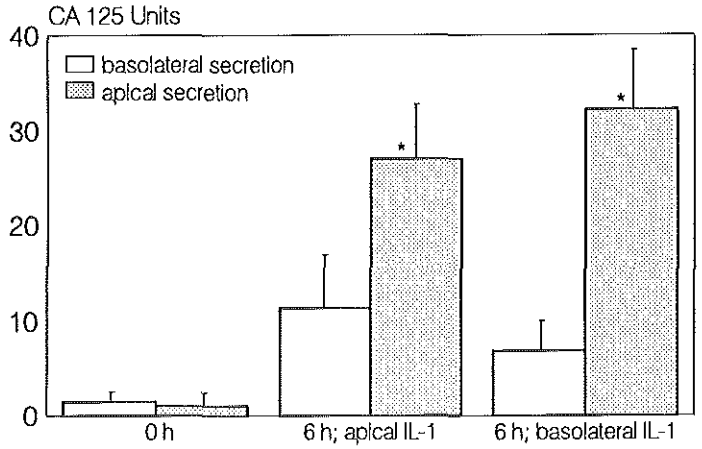

Figure 1. Apical and basolateral secretion of CA 125 by confluent monolayers of mesothelial cells. Stimulation of the mesothelial cells took place by adding rIL-1β (25 U/ml) at 0 hours to the apical or basolateral side of the monolayer. Results are expressed as the mean (SEM) in units of five separate experiments. *Apical secretion was significantly higher compared with the basolateral secretion of CA 125 at the same timepoint. ($p \leq 0.05$; two tailed paired Student *t*-test).

For six hours, mean (SD) 17.9 (4.1) units of CA 125 were secreted by cultured human mesothelial cells without deliberate activation of the cells with inflammatory stimuli. All stimuli used for the mesothelial cell monolayers induced a noticeable increase in the secretion of CA 125 into the supernatant fluid. The most effective stimulus of mesothelial cell CA 125 secretion was seen with rIL-1β; a 77% increase was noted when rIL-1β was present in the incubation mixture (Figure 2).

DISCUSSION

Cancer antigen 125 is a highly sensitive tumormarker for ovarian epithelial tumors: OC 125, the antibody against CA 125, can recognise all of six human ovarian cancer cell lines.[1] In spite of the fact that OC 125 fails to react with normal ovary cell lines, and with 13 out of 14 non-ovarian cancer cell lines, the specificity of CA 125 is remarkably low. Increased concentrations of serum CA 125 have been found in patients with liver cirrhosis, hepatocellular carcinoma, and tuberculous peritonitis.[7 8 12] Increased serum CA 125 concentrations are also seen after abdominal surgery.[6] Increased serum CA 125 concentrations have likewise been detected in women during the menstrual cycle, pregnancy, and during pelvic inflammatory disease.[13 14] The results of the present study suggest that increased serum CA 125 concentrations in all the cases mentioned may have their origin in activated peritoneal mesothelium.

Mesothelium has sophisticated junctional complexes that might allow a gradient of

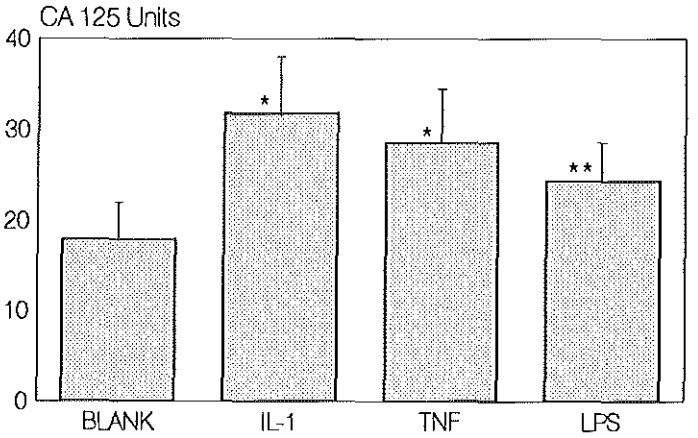

Figure 2. Influence of inflammatory stimuli on apical CA 125 secretion of mesothelial cells. Stimulation of the mesothelial cells took place by adding rIL-1β (25 U/ml), TNF-α (100 U/ml) or LPS *E. coli* (1 µg/ml) at 0 hours. CA 125 secretion was measured six hours later. Results are expressed as the mean (SEM) units CA 125 based on four separate experiments. *CA 125 secretion was significantly higher compared with the secretion of unstimulated MC at 6 hours. (*p≤0.05; **p≤0.01; two tailed paired Student *t*-test).

CA 125 to be created.[15] Other workers have stated that the peritoneum serves as a barrier for high molecular weight tumor antigens such as CA 125, as CA 125 values in the ascites of patients with ovarian carcinoma were found to be up to 130 times higher compared with those in the serum.[16 17] In our in vitro model the apical side of the mesothelial cell monolayer correlates with the side facing the abdominal cavity in vivo. Our results indicate that CA 125 is preferentially secreted towards the abdominal cavity. These results further support the concept that peritoneum indeed forms a barrier for CA 125 transport and contributes actively to the CA 125 gradient accross the peritoneal membrane. It is likely that CA 125 reaches the circulation through lymphatic absorption via the large fenestrae present in the diafragmatic peritoneum.

Polarized secretion of proteins by a cultured monolayer of mesothelial cells is not unique to CA 125: Interleukin-8, a chemoattractant for neutrophils[18], is also produced by mesothelial cells and is likewise secreted preferentially via the apical surface of activated mesothelial cells. This gradient of interleukin-8 is probably of paramount importance in the migration of neutrophils through the mesothelial cell monolayer towards the peritoneal cavity.[19]

The active role of mesothelium in inflammatory processes during bacterial and non-bacterial (carcinomatous) peritonitis is not yet fully understood. However, the present data show that CA 125 can no longer be seen only as a tumor marker. Further investigations are recommended to delineate the role of CA 125 production by mesothelial cells.

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9

GENERAL DISCUSSION AND SUMMARY

The adherence to and subsequent migration of leukocytes across mesothelium are key events in the inflammatory response during peritonitis. This thesis describes a number of regulatory mechanisms that characterize the basic processes of neutrophil and monocyte adherence to and migration across activated human peritoneal mesothelial cells in an *in vitro* model of peritonitis.

Chapter 1 describes both macroscopic and microscopic properties of the mesothelium, with emphasis on the characteristic changes during peritonitis. One of the main aspects of this thesis is the study of leukocyte migration across cultured mesothelium. In general, the leukocyte influx from the bloodstream towards the inflammatory tissue is guided by chemoattractants. These 'attracting' substances may originate from different sources, such as bacteria, complement and different types of cell, including endothelium. Some of the chemoattractants involved in leukocyte migration across endothelium are described. Before migration can take place, leukocytes must adhere avidly to endothelial cells. The last part of the introduction summarizes the current state of knowledge concerning the specific adhesion receptors on both leukocytes and endothelial cells involved in adherence to each other, thus describing the regulation of the first steps of the migration of these leukocytes in leaving the circulation in inflammatory events.

One of the major neutrophil chemoattractants is interleukin-8 (IL-8), which can be synthesized by a large variety of cells (including mesothelium). IL-8, may thus, play a crucial role in different inflammatory processes throughout the human body. The functional role of IL-8 secretion by mesothelial cells is described in **Chapter 2**. The secretion pattern of IL-8 by activated mesothelial cell monolayers showed a polarized secretion of IL-8, preferentially towards the apical side of the monolayers. This means, *in vivo*, that in the abdominal cavity more IL-8 is present than on the other side of the mesothelial monolayer, i.e. in the submesothelial tissue. Neutrophils are capable of migrating towards the highest concentration in a gradient of chemoattractants (the exact mechanism of this process is not known). The polarized secretion pattern of IL-8 by the mesothelial monolayers concurs with the physiological situation, since the infection in peritonitis takes place at the apical side of monolayer (i.e. the abdominal cavity). The functional role of the polarized secretion by IL-8 was quantitated through assays of PMN migration across activated mesothelial monolayers. For this purpose, mesothelial cell monolayers were cultured 'hanging' on filters, to study the migration in the physiological direction (i.e. submesothelial tissue to abdominal cavity). This assay system, thus, permitted the neutrophils to approach and adhere to the basolateral side of the monolayer. A significantly increased migration of PMN was observed in this direction, compared to the migration across mesothelial monolayers cultured not 'hanging' from but 'lying down' on top of the filters. The importance of IL-8 in this

process was shown by reduction of PMN migration across inverted activated monolayers (63%) in the presence of specific antibodies against IL-8.

Chapter 3 describes the neutrophil adherence to and migration across resting and IL-1β-pretreated mesothelial cell monolayers in more detail. The involvement of mesothelium adhesion receptor ICAM-1 and of the neutrophil receptor CR3 were defined, as were the circumstances under which adherence occurs. It is concluded that neutrophil activation by IL-1β-pretreated mesothelial cells is necessary before adhesion takes place. This step requires *de novo* protein synthesis by the mesothelial cells.

In this thesis we have described the secretion of IL-8 by activated mesothelial cells both *in vivo* and *in vitro*. We found in **Chapter 4** that the abdominal fluid of patients suffering from appendicitis with a perforated appendix contained more IL-8, than fluids harvested from patients with non-perforated appendicitis, indicating an important role for bacteria in inducing the inflammatory cascade. Anaerobic bacterial species, such as *Bacteroïdes*, which are frequently isolated in surgical peritonitis and aerobic species, such as *Staphylococcus*, which frequently cause CAPD-related peritonitis, were found to induce cultured mesothelial cells to secrete large amounts of IL-8. However, additional regulatory mechanisms of neutrophil influx may be present in the peritoneal cavity during peritonitis in an *in vivo* situation. One possible mechanism is the processing of IL-8. Thrombin[1] and elastase[2] are able to convert IL-8 from a 77-amino-acid into an 72-amino-acid protein, which changes IL-8 into a more active chemoattractant.[3] We confirmed this reaction *in vitro*: the IL-8 secreted by mesothelial cells is, at least in part, the 77-amino acid form of IL-8. Thrombin further increased the chemotactic capacity of supernatant obtained from activated mesothelial cells (unpublished data). Thus, blood spill into the peritoneal cavity during peritonitis, e.g. during surgical intervention, causes activation of the coagulation system, subsequently followed by thrombin release and thus, may stimulate the PMN influx if IL-8 is also present. Another pathway of splicing IL-8 in this cascade is auto-activation of the neutrophil influx by elastase; this toxic enzyme is released by the PMN themselves after degranulation of the azurophilic granules.

It can be concluded from the previous chapters that the onset of the neutrophil influx during peritonitis is a localized process in which mesothelium plays a key role. The prevailing concept that monocytes are the main source of IL-8 production during the beginning of peritonitis[4] must therefore be reconsidered. Modulation of the neutrophil influx during inflammation has been proven beneficial in animal studies. Whenever modulation of the PMN influx during peritonitis is wanted, it would be preferable to do so at the very beginning of the influx, e.g. by giving mAb

against IL-8 intraperitoneally. Unfortunately, at present it is impossible to predict which patients with peritonitis are likely to develop a multiple organ failure despite antibiotic and surgical therapy. Further research in this field may lead to new strategies of modulating the neutrophil influx into the peritoneal cavity as an adjunct to the conventional therapy for peritonitis.

Chapter 5 describes the opsonin transport across cultured mesothelial cells. Mesothelial monolayers are a relative barrier for opsonin transport. However, whenever neutrophils migrate across the cell layer, opsonins seem to 'travel' with them. It is likely that during the PMN migration the confluence of the monolayer decreases, so that proteins, such as opsonins, can diffuse passively to the abdominal cavity and thereby enhance the elimination of the bacteria.

In **Chapter 6**, activation of peripheral blood and peritoneal effluent neutrophils was studied in patients with CAPD-associated peritonitis. The surface antigen expression of the peritoneal exudate PMN, isolated from the first 'cloudy bag,' had changed compared to PMN isolated from the blood of the same patient: an increased expression of early activation markers was observed. However, degranulation of the azurophilic granules could not be detected. These changes in granulocyte activation are not unique for peritonitis: it was previously observed in PMN isolated from joint fluid of patients suffering from arthritis. Apoptosis, also known as 'programmed cell death' was not measured in the exudate PMN during the initial phase of peritonitis. Furthermore, a difference in chemotactic response between the blood or peritoneal neutrophils towards the chemoattractants FMLP, PAF, C5a, or IL-8 was also not observed.

Most of the studies described in this thesis deal with neutrophil interactions with mesothelial cells. The influence of the mesothelium in *monocyte* adherence to and migration across monolayers of mesothelium was also studied, the results of which are summarized in **Chapter 7**. The chemotactic response of monocytes towards activated mesothelial supernatant and modulation of this response by mAbs against MCP-1 and TGF-β were measured by the Boyden-chamber technique. The general conclusion of this chapter is that, in sharp contrast to neutrophil influx, monocyte influx across cultured mesothelium is not modulated after activation of mesothelium at all.

Chapter 8 describes the secretion of CA 125 by activated mesothelial cells. CA 125 is generally used as a tumour marker for ovarium carcinoma.[5] Earlier histological studies have shown the presence of CA 125 at the surface of peritoneal mesothelium,[6] suggesting active participation of these cells in CA 125 synthesis. Furthermore, increased levels of CA 125 have been found in sera of

patients during episodes of bacterial peritonitis.[7,8] Indeed, we found that cultured mesothelial cells are able to secrete CA 125 whenever activated by various pro-inflammatory products, such as IL-1β, TNF or LPS from *E. coli*. In future, CA 125 can be used not only as a tumour marker in the follow up of ovarian carcinoma, but also as a marker for processes in which activation of the mesothelium takes place.

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NEDERLANDSE SAMENVATTING

De binding en hieropvolgende migratie van witte bloedcellen (leukocyten) door het buikvlies (mesotheel) zijn centrale gebeurtenissen in de ontstekingsrespons die optreedt tijdens buikvliesontsteking (peritonitis). Dit proefschrift beschrijft verscheidene regulatiemechanismen die het proces van aanhechting en migratie van neutrofiele granulocyten en monocyten door humane peritoneale mesotheelcellen karakteriseren, bestudeerd in een *in vitro* model voor peritonitis.

In **Hoofdstuk 1** worden de macroscopische en microscopische eigenschappen van mesotheel beschreven, met nadruk op karakteristieke veranderingen die optreden tijdens peritonitis. In dit proefschrift is vooral de migratie van leukocyten door een laag van gekweekte mesotheelcellen bestudeerd. Over het algemeen wordt de influx van leukocyten vanuit de bloedbaan naar ontstoken weefsels gemedieerd door zogenaamde chemoattractantia. Deze leukocyten aantrekkende stoffen kunnen van zeer uiteenlopende oorsprong zijn (bacterie of complement) of geproduceerd worden door verschillende celtypen, waaronder endotheelcellen. In dit hoofdstuk worden een aantal chemoattractantia, die een rol spelen in de migratie van leukocyten door endotheel, beschreven. Voordat migratie plaats kan vinden moeten leukocyten eerst binden aan specifieke receptoren op de endotheelcellen. In het laatste gedeelte van dit hoofdstuk wordt de hedendaagse kennis met betrekking tot deze specifieke adhesie receptoren op zowel leukocyten en endotheelcellen samengevat. Hiermee wordt de eerste stap van het proces bechreven, waarmee leukocyten de bloedsomloop verlaten tijdens ontstekingen.

Een van de belangrijkste chemoattractantia voor neutrofiele granulocyten is het interleukine-8 (IL-8), dat gesynthetiseerd kan worden door verschillende celtypen (waaronder mesotheel). IL-8 zou daarom een cruciale rol kunnen spelen in diverse ontstekingsprocessen op verschillende plaatsen in het menselijk lichaam. In **Hoofdstuk 2** wordt de secretie van IL-8 door mesotheelcellen beschreven. Het blijkt dat de secretie van IL-8 door een monolaag van geactiveerde mesotheelcellen gepolariseerd plaatsvindt, met een voorkeur voor de apicale kant de van de monolaag. Dit betekent dat *in vivo* meer IL-8 aanwezig zal zijn in de buikholte dan aan de andere kant van de mesotheel monolaag (het submesotheliale weefsel). Neutrofiele granulocyten hebben de eigenschap zich in een gradient van chemoattractantia voort te bewegen naar de plek met de hoogste concentratie. De gepolariseerde secretie van IL-8 is in overeenstemming met de fysiologische situatie, aangezien de ontsteking tijdens peritonitis plaatsvindt aan de apicale zijde van de mesotheel monolaag. In dit hoofdstuk werd de gepolariseerde secretie van IL-8 door mesotheelcellen ook bestudeerd in een functionele assay. In deze assay werd

de migratie van neutrofiele granulocyten door een monolaag van geactiveerde mesotheelcellen gemeten. Een speciale kweektechniek werd ontwikkeld, waarbij mesotheelcellen hangend aan filters werden gekweekt, zodat migratie bestudeerd kon worden in een fysiologische richting (van de submesotheliale kant naar de intra-abdominale kant), doordat neutrofiele granulocyten de basolaterale kant van de monolaag konden naderen. Een significant toegenomen migratie van neutrofiele granulocyten werd gemeten op deze wijze ten opzichte van mesotheelcel monolagen die op de confentionele manier waren gekweekt (niet hangend aan, maar liggend op het filter en dus in de verkeerde richting). De migratie bleek IL-8 afhankelijk te zijn, aangezien de aanwezigheid van een IL-8 remmend antilichaam de migratie door omgekeerd gekweekte mesotheelcellen voor 63% kon remmen.

In **Hoofdstuk 3** wordt de aanhechting van neutrofiele granulocyten aan en de migratie door ongeactiveerde en met IL-1β voorbehandelde mesotheel monolagen beschreven. De rol van de adhesie receptoren ICAM-1 op de mesotheelcel en CR3 op de neutrofiele granulocyt werd bepaald en de omstandigheden waaronder adhesie plaatsvindt werd onderzocht. Er werd geconcludeerd dat activatie van de neutrofiele granulocyt door IL-1β voorbehandelde mesotheelcellen nodig is om adhesie te doen plaatsvinden. Voor deze stap is *de novo* eiwit synthese door de mesotheelcel nodig.

In dit proefschrift hebben we niet alleen secretie van IL-8 door mesotheelcellen in vitro, maar ook in vivo aangetoond. In **Hoofdstuk 4** lieten we zien dat in patiënten met appendicitis, die een geperforeerde appendix hadden, meer IL-8 aanwezig was in het abdominale vocht dan in patënten met appendicitis en een niet geperforeerde appendix. Ook werd aangetoond dat niet alleen anaërobe bacteriën, zoals *Bacteroïdes*, die vaak geïsoleerd worden bij peritonitis, maar ook aërobe bacteriën, zoals *Staphylococcus*, die vaak CAPD-gerelateerde peritonitis veroorzaken, mesotheelcellen direct kunnen aanzetten tot secretie van IL-8. Deze resultaten veronderstellen een belangrijke rol voor bacteriën in het aanzetten van ontstekingsreacties. Echter, *in vivo* bestaan er waarschijnlijk nog andere mechanismen om de influx van neutrofiele granulocyten te reguleren. Een belangrijk mechanisme is waarschijnlijk de omzetting van IL-8 in activere vormen. Trombine en elastase kunnen IL-8 omzetten van een 77 in een 72 aminozuur lang eiwit, waardoor het een veel sterker werkend chemoattractant wordt. Deze reactie werkte ook in ons *in vitro* systeem: de chemotactische capaciteit van supernatant van geactiveerde mesotheelcellen kon worden verhoogd door thrombine (niet gepubliceerde data). Dit betekent dat wanneer er tijdens een operatie wegens peritonitis, bloed terecht komt in de peritoneale holte de na stolling gevormde trombine de influx van neutrofiele

granulocyten zou kunnen versterken. Overigens het feit dat IL-8 ook door elastase geknipt kan worden betekent dat de influx van neutrofiele granulocyten zichzelf ook kan versterken: dit enzym komt vrij uit de azurofiele granulae van geactiveerde neutrofiele granulocyten.

Op basis van de gegevens gepresenteerd in de vorige hoofdstukken kan geconcludeerd worden dat de influx van neutrofiele granulocyten tijdens peritonitis een gelokaliseerd proces is waarin het mesotheel een centrale rol speelt. Het heersende concept dat monocyten de belangrijkste bron zijn van IL-8 tijdens het ontstaan van een peritonitis, moet daarom worden herzien. Met behulp van dierproeven is aangetoond dat het moduleren van de influx van neutrofiele granulocyten nuttig kan zijn. Als men de influx van neutrofiele granulocyten tijdens peritonitis zou willen moduleren, zou dat het best gedaan kunnen worden door te interfereren bij het op gang komen van de influx, door bijvoorbeeld monoclonale antilichamen tegen IL-8 intraperitoneaal te geven. Echter, het is op dit moment nog onmogelijk om te voorspellen welke patiënten systemisch orgaan falen zullen gaan ontwikkelen ondanks antibiotische en chirurgische therapie en dus mogelijk baat hebben bij modulatie van de influx van neutrofiele granulocyten. Toekomstig onderzoek in dit gebied zal misschien resulteren in nieuwe methoden om de influx te moduleren, die mogelijk een belangrijke toevoeging kunnen zijn aan de conventionele therapie.

In **Hoofdstuk 5** wordt het opsoninen transport door mesotheelcel monolagen beschreven. Deze monolagen bleken een relatieve barriere voor het opsoninen transport te zijn. Echter, tijdens het transport van neutrofiele granulocyten door de cellaag verhoogt ook het transport van opsoninen. Waarschijnlijk vermindert tijdens migratie de confluentie van de monolaag, waardoor eiwitten, zoals opsoninen, passief in de abdominale holte kunnen diffunderen en daar de eliminatie van bacteriën kunnen versterken.

In **Hoofdstuk 6** wordt de activatie van neutrofiele granulocyten uit het perifere bloed en uit het peritoneale effluent (de eerste troebele zak) van patiënten met CAPD geassocieerde peritonitis bestudeerd. De expressie van oppervlakte antigenen van neutrofielen geïsoleerd uit het effluent verschilde van neutrofielen geïsoleerd uit het bloed van dezelfde patiënt. Alhoewel de vroege activatie markers een verhoogde expressie vertoonden, kon er geen degranulatie van azurofiele granulae gedetecteerd worden. Deze verschillen in granulocyt activatie zijn niet uniek voor peritonitis: in neutrofiele granulocyten geïsoleerd uit gewrichtsvloeistof van arthritis patiënten is deze activatie ook gevonden. Apoptose (geprogrammeerde celdood) kon niet aangetoond worden in de neutrofielen in de initiële peritonitis fase. Ook vertoonden de neutrofiele

granulocyten uit het buikvocht en het bloed geen verschillen in chemotactische respons jegens de chemoattractantia FMLP, PAF, C5a en IL-8.

Het leeuwendeel van de studies beschreven in dit proefschrift gaat over de interactie tussen mesotheelcellen en neutrofiele granulocyten. De rol die het mesotheel speelt in de adhesie van monocyten aan mesotheelcellen en de hieropvolgende migratie werd onderzocht in **Hoofdstuk 7**. De chemotactische respons van monocyten jegens supernatant van geactiveerde mesotheelcellen en de modulatie van deze respons door antilichamen tegen MCP-1 en TGF-β werden bepaald met de Boyden-kamer techniek. Geconcludeerd werd dat, in tegenstelling tot neutrofiele granulocyten, de influx van monocyten door mesotheel totaal niet gemoduleerd wordt na activatie van mesotheelcellen.

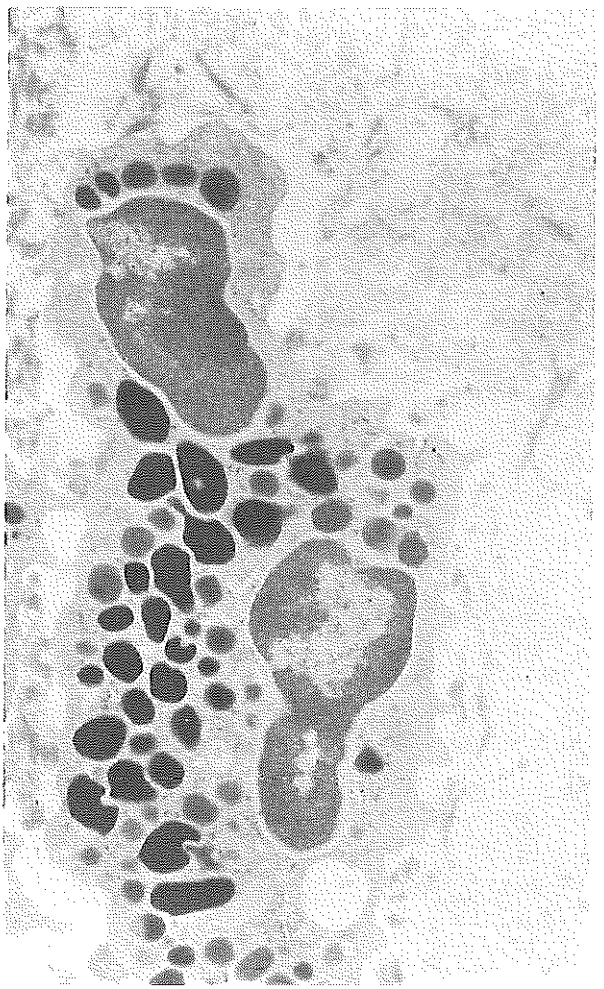
In **Hoofdstuk 8** wordt de secretie van CA 125 door geactiveerde mesotheelcellen beschreven. CA 125 wordt gewoonlijk gebruikt als een tumor marker voor ovarium carcinomen. Eerdere histologische studies hebben laten zien dat CA 125 aanwezig is op het oppervlak van peritoneale mesotheelcellen, wat erop wijst dat deze cellen CA 125 kunnen synthetiseren. Verder zijn verhoogde spiegels van CA 125 gevonden in sera van patienten met bacteriële peritonitis. Wij vonden dat gekweekte mesotheelcellen CA 125 kunnen uitscheiden als ze geactiveerd worden door ontstekingsmediatoren, zoals IL-1β, TNF of LPS van *E. coli*. Dit betekent dat CA 125 niet alleen als tumor marker in de follow up van ovarium carcinomen gebruikt kan worden, maar ook als marker voor mesotheel activatie.

CURRICULUM VITAE

Anneke Zeillemaker werd op 23 december 1962 geboren te Almelo. Zij behaalde het diploma HAVO in 1980 en het diploma VWO in 1982 aan het Christelijk Lyceum in Almelo. Vanaf augustus 1982 tot juli 1983 werkte zij als au-pair in New York, waar zij tevens Frans studeerde aan de Alliance Française. In 1983 begon zij met de studie Geneeskunde te Utrecht, alwaar in december 1991 het arts-examen werd behaald.
Van februari 1992 tot februari 1995 was zij als arts/onderzoeker in dienst van de Stichting Wetenschappelijk Onderzoek Diakonessenhuis Utrecht (SWODU) waar dankzij een subsidie van de Nierstichting Nederland het onderzoek werd verricht dat resulteerde in dit proefschrift, onder leiding van Dr P. Leguit, Dr A.A.G.M. Hoynck van Papendrecht en Prof. Dr H.A. Verbrugh (thans Instituut Klinische Microbiologie, Erasmus Universiteit, Rotterdam). In deze periode werden ook experimenten verricht op de afdeling Bloedcelchemie van het Centraal Laboratorium van de Bloedtransfusiedienst van het Nederlandse Rode Kruis (CLB) te Amsterdam, onder leiding van Prof. Dr D. Roos.
In februari 1995 werd begonnen met de opleiding tot chirurg in het Academisch Ziekenhuis te Utrecht (opleider: Prof. Dr Th.J.M.V. van Vroonhoven).

CALIFORNIA BIGFOOT

An electron micrograph shows a neutrophilic leukocyte from an acute bacterial inflammatory reaction in the peritoneal cavity of a rabbit. Two segments of the nucleus can be seen, as well as a cytoplasm full of granules that contain antibacterial and lysosomal enzymes (x12,000).



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