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Acute inflammation in peritoneal dialysis : experimental studies in rats

Characterization of regulatory mechanisms

Farhan Bazargani

Göteborg 2005

ABSTRACT

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Characterization of regulatory mechanisms

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The predominant problems associated with peritoneal dialysis (PD) are ultrafiltration failure and peritonitis. PD maintains a state of intraperitoneal inflammation that affects the structure and function of the peritoneal membrane, potentially impairing ultrafiltration efficiency. Paradoxically, some PD fluids also have anti-inflammatory properties that may compromise the immune defense against peritonitis. This anti-inflammatory feature is mostly due to the glucose degradation products (GDPs), formed during heat-sterilization and storage of PD fluids. The main purpose of the present thesis was to study regulatory mechanisms behind the acute intraperitoneal inflammatory response in PD in the presence and absence of experimental peritonitis.

Rats were exposed to a single dose of heat- or filter sterilized PD fluids either as an i.p. injection or as an infusion through an indwelling catheter, with or without supplementations, or pretreatment of the animals. The dwell fluid was analyzed zero, two and four hours later concerning activation of the complement and coagulation cascades, neutrophil recruitment and respiratory burst, ultrafiltration volumes, cytokine-induced neutrophil chemoattractant (CINC-1), rat mast cell protease 2 (RMCP-2), glucose, urea and histamine concentrations and *ex vivo/in vitro* intraperitoneal chemotactic activity.

Exposure to filter sterilized PD fluid alone induced intraperitoneal complement activation and coagulation, neutrophil recruitment and increased the levels of CINC-1 during the dwell. Intraperitoneal concentrations of the mast cell markers histamine and RMCP-2 changed little during the dwells and did not indicate mast cell activation. Low molecular weight heparin (LMWH) and C5 blockade improved ultrafiltration. Pretreatment with cobra venom factor, known decomplementing agent, blocked the CINC-1 release and the neutrophil recruitment and improved ultrafiltration. In combination with experimental peritonitis, heat sterilized PD fluid compared to filter sterilized, inhibited the CINC-1 release and the recruitment of neutrophils to the peritoneal cavity without affecting the intraperitoneal complement activation.

The results of the present thesis indicate that addition of LMWH to the PD fluid improves ultrafiltration, probably by blocking C5a activity. C5 blockade seems to improve ultrafiltration by a mechanism that involves a reduction in glucose transport, possibly by reducing C5 induced vasodilation. Complement activation is an early step in the acute reaction to PD and probably mediates the downstream events that lead to the recruitment of inflammatory cells to the peritoneal cavity. The cells involved in the release of CINC-1 later in this sequence are probably the mesothelial cells. During experimental peritonitis, heat sterilized PD fluids inhibited the neutrophil respiratory burst response of intraperitoneal neutrophils. Heat sterilized PD fluids also inhibit the recruitment of neutrophils to the peritoneal cavity by a mechanism independent of complement activation but probably depending on cytokine CINC-1 release during peritonitis.

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Low molecular weight heparin improves peritoneal ultrafiltration and blocks complement and coagulation. *Perit Dial Int*. 2004 In press

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The roles of complement factor C5a and CINC-1 in glucose transport, ultrafiltration and neutrophil recruitment during peritoneal dialysis.
Perit Dial Int Submitted

III. **Bazargani, F. and Braide, M.**
PD fluid induced CINC-1 release and neutrophil recruitment is mediated by complement activation. Manuscript

IV. **Jonasson, P., Bazargani, F., Braide, M.**
Heat-sterilized PD fluid has differential effects on mechanisms behind neutrophil recruitment and responsiveness in experimental peritonitis.
Asaio J Submitted

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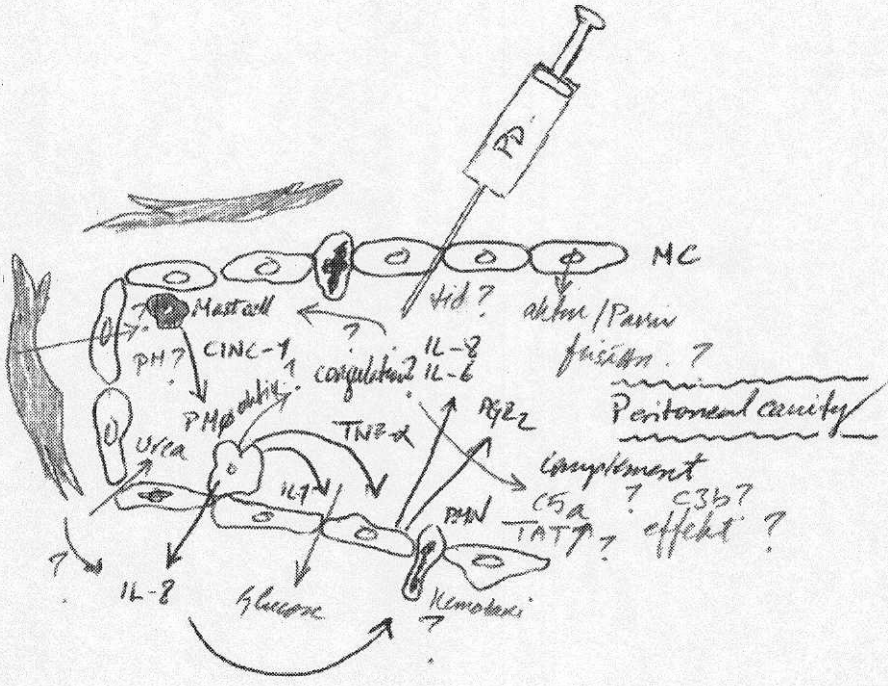
Institute of Anatomy and Cell Biology **Faculty of Odontology**

The Sahlgrenska Academy at Göteborg University
Sweden, 2005

To Silvia & Samuel, with love



The never failing "insight of animal experiments":

"Causes of death seem simpler than causes of life;
Death can be caused in many reproducible ways"

L. Vroman, 1971

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CONTENTS

LIST OF PAPERS iii

ABBREVIATIONS iv

INTRODUCTION 1

AIMS 14

MATERIALS AND METHODS 15

RESULTS 25

DISCUSSION 40

CONCLUSSIONS 48

ACKNOWLEDGEMENTS 49

REFERENCES 51

APPENDIX: PAPERS I - IV

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I – IV).

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ABBREVIATIONS

BSA	bovine serum albumin
CAPD	continuous ambulatory peritoneal dialysis
CINC-1	cytokine-induced neutrophil chemoattractant-1
CVF	cobra venom factor
C5	complement factor 5
DMSO	di-methyl-sulf-oxide
EDTA	ethylene-diamine-tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
f-MLP	formyl-methionyl-leucyl-phenylalanine
GDP	glucose degradation product
HBSS	Hank´s balanced salt solution
IL	interleukin
LTB_4	leukotriene B_4
LMWH	low molecular weight heparin
NO	nitric oxide
PD	peritoneal dialysis
PBS	phosphate-buffered saline
RMCP-2	rat mast cell protease 2
TAT	thrombin-antithrombin complex
TNF-α	tumor necrosis factor alpha
TXB_2	thromboxane B_2

INTRODUCTION

Peritoneal dialysis

Continuous ambulatory peritoneal dialysis (CAPD) is a treatment for uremic patients with end-stage renal failure. In this treatment, the peritoneal cavity is repeatedly filled with a hypertonic peritoneal dialysis (PD) fluid through a permanent catheter implanted in the abdominal wall, and then drained, usually after 4-6 hours of dwell time. During CAPD, the peritoneal membrane is used as an endogenous dialyzing membrane to remove metabolic waste products (primarily urea and creatinine) and water from the blood circulation to the peritoneal cavity by a combination of diffusion and osmosis [1, 2].

Peritoneal dialysis was first introduced in patients with acute renal disease. With the development of more sophisticated methods for infusion of PD fluids, the treatment could also be used on patients with chronic renal disease. Since CAPD was first used almost 25 years ago, it has become an alternative to haemodialysis for treatment of patients with end-stage renal failure. However, the predominant problems with CAPD remain. These are a high incidence of peritonitis, and ultrafiltration failure [3].

Peritonitis remains a major complication of PD, accounting for much of the morbidity associated with the technique. The most common organisms producing peritonitis are Gram-positive and include mainly *Staphylococcus epidermidis* and *Staph. aureus* [4]. With declining rates of Gram-positive infection secondary to changes in catheter connection technique, the proportion of infections secondary to Gram-negative organisms is increasing. In most cases, Gram-negative peritonitis is caused by intestinal flora. This can result from abdominal perforation, instrumentation, or other abdominal processes. However, in many cases, an etiology of the infection is not found [5]. Peritonitis accounts for 15-35% of hospital admissions and is the major cause of transfer to haemodialysis [6].

Ultrafiltration occurs in PD when there is net fluid filtration into the peritoneum. For osmotically driven ultrafiltration to occur, a semi-permeable membrane must separate two physiological compartments, with one of the compartments having an effective osmolality to induce fluid shift. The increased osmolality of hypertonic

glucose-containing PD solutions produces an osmotic gradient, which favors fluid movement from the vascular compartment to the peritoneal cavity [7]. The capillary membrane has the physiological characteristics of a semi-permeable membrane. The microcirculation at the peritoneal surface is exposed to the hyperosmotic effects of PD solutions via the mesothelial lining and the submesothelial connective tissue.

Numerous endogenous and exogenous vasoactive agents have been shown to modify blood flow in the peritoneal microcirculation. A wide variety of drugs, hormones, neurotransmitters, and mediators of inflammation alter mesenteric vascular resistance. In general, vasodilators in the splanchnic circulation enhance peritoneal clearance and increase capillary filtration coefficients, while vasoconstrictors decrease peritoneal clearance and reduce capillary filtration coefficients [8]. Diffusion of small molecules (e.g., urea and glucose) is dependent on the capillary blood flow during PD [9]. An increase in capillary flow would increase the out-diffusion of substances such as glucose, which is the most common osmotic agent in PD fluids. Filtration and large molecule transcapillary transport is not blood flow-dependent and an increase in capillary flow may therefore lead to rapid disappearance of the osmotic gradient and, consequently, termination of the ultrafiltration process. While no broad generalization regarding the relationship between vasoactive agents and permeability may be made, it should be noted that many vasodilators also act to increase microvascular permeability [10]. For example, bradykinin and histamine increase both blood flow and permeability [11, 12].

In maintaining the ultrafiltration and solute clearance, preservation of the peritoneal tissue is of great importance. Despite a great amount of work having been performed to investigate the bioincompatible effects of the different components of the PD fluids on the peritoneal cells and tissue, the composition of the PD fluids has remained more or less unchanged over the years.

Composition of peritoneal dialysis fluids

The components of the PD fluids are generally an osmotic agent, electrolytes, and a buffer solution. As osmotic agent, glucose is used, mainly because it is cheap and easily metabolized. The drawbacks of using glucose are rapid absorption (and, consequently, short ultrafiltration time), the exposure of the patient's metabolism to a

large amount of carbohydrate, and instability during heat sterilization and storage [2], which contributes to formation of toxic glucose degradations products (GDPs).

The composition of electrolytes (Na^+, Cl^-, Ca^{2+}, and Mg^{2+}) is similar to that found in blood plasma. However, it has been suggested that low sodium concentrations in dialysate may be beneficial for patients with overhydration and may prevent cardiovascular problems [13].

The physiological buffer in the body is bicarbonate. However, the use of bicarbonate in PD fluids may cause precipitation of calcium carbonate during storage of carbonate buffer solutions. Currently, lactate is the most commonly used buffer in PD fluids. Lactate is present in human plasma; however, the levels of lactate ion in PD fluid are up to 80 times higher than those found in human plasma [14]. Recent studies have shown that lactate-containing PD fluids induce pronounced morphological changes of the peritoneum, such as neoangiogenesis and mild damage to mesothelial cells [15]. Since pyruvate-based PD solutions have been shown to be more biocompatible than lactate-based ones, pyruvate has been suggested as a buffer of choice [16].

Structure and function of the peritoneum

The peritoneal cavity contains a small amount of fluid (about 0.5–1.0 dl), in which different types of leukocytes, principally monocytes/macrophages (90%), neutrophils (5%), and lymphocytes (5%), are suspended. The main phagocytes of the normal peritoneal cavity are not polymorphonuclear leukocytes, but monocytes/macrophages [17]. Plasma proteins are present at concentrations approximately equal to those normally found in plasma. The peritoneal cavity is lined with a monolayer of mesothelial cells. These are formed in a cobblestone layer and are covered with surface microvilli. Besides a high synthetic activity, the main function of the mesothelium is lubrication of the peritoneal wall and protection of the submesothelial tissue from exposure to invading microorganisms and toxic products.

The submesothelial tissue is separated from the mesothelium by a basal membrane. It contains collagen fibers in a matrix of ground substance. Embedded in the tissue are cells, blood capillaries, and lymphatics. Normally, only a few cells are present, mostly fibroblasts, mast cells, lymphocytes, and macrophages. The microcirculation

of the peritoneal membrane provides the site for physiological interaction and exchange between the circulating blood and the cells and fluid of the peritoneal cavity [8].

The inflammatory response of the peritoneum

The resident defending cells of the peritoneum are located at four different sites: in the submesothelial connective tissue, in the submesothelial perivascular unit, suspended in the peritoneal fluid, and adherent to the peritoneal lining. Macrophages, mesothelial cells, lymphocytes, mast cells, and opsonins of the peritoneal cavity play an important role in the defense of the human host against bacterial pathogens. The principal opsonins in the fluid in the peritoneal cavity, as in plasma, are IgG, complement components, and fibronectin [2, 17].

Following a bacterial infection of the peritoneal cavity, the initial reaction (i.e., within the first 2 hours) is activation of the plasma system (complement, kinin, and coagulation systems) and the intraperitoneal (i.p.) macrophages. Activation of the complement system may lead to opsonization of the invading bacteria, thus preparing their recognition by phagocytes and chemotaxis, resulting in neutrophil migration. Activation of macrophages by the plasma systems and by opsonized or unopsonized microorganisms results in secretion of both prostaglandins (TXB_2) and PGE_2) and cytokines (IL-1β,IL-1α, TNF-α, andIL-8). As a consequence, mesothelial cells start to synthesize prostaglandins and cytokines, in particular PGE_2 and PGI_2, together with IL-1β and IL-8. Of these, IL-8 is a powerful chemoattractant for polymorphonuclear cells [18-20].

The dominating cell type recruited by the acute inflammatory response to a bacterial and fungal infection is the neutrophils from the venules of the peritoneal microcirculation. An increase in the neutrophil fraction of the PD effluent is commonly seen as an early diagnostic criterion for bacterial peritonitis [21]. Inflammatory stimuli such as thrombin released by the coagulation system, complement products (e.g., C5a), and various cytokines (e.g., TNF-α and IL-8) activate the endothelium of the venules. The activation leads to expression of adhesion molecules, which mediate adhesion and activation of neutrophils and other leukocytes. The cells reversibly adhere to the endothelium and roll slowly along the vascular wall, predominantly in the small venules. Glycoprotein molecules called

selectins mediate this reversible adhesion, this being the first step in the recruitment of neutrophils. The selectin that is constitutively expressed on the surface of leukocytes, L-selectin, interacts with the endothelium selectins P-selectin and E-selectin (see Fig. 1, top panel; P-selectin not shown). This interaction is highly coordinated and complex. P-selectin, which is carried inside endothelial cells in granules known as "Weibel-Palade bodies", appears on endothelial cell surfaces within a few minutes of exposure to LTB4, C5a, or histamine. The second selectin, E-selectin, appears a few hours after exposure to lipopolysaccharide or TNF-®. The interaction of P-selectin and E-selectin with L-selectin allows neutrophils to adhere reversibly and to *roll along* the vessel wall. This adhesive interaction permits the stronger interaction of the second step in the leukocyte recruitment.

The second step in neutrophil recruitment depends upon interactions between the neutrophil integrins known as LFA-1 (CD11a/CD18) and MAC-1 (CD11b/CD18) and molecules on endothelium, such as the immunoglobulin-related intercellular adhesion molecule 1 (ICAM-1), which is also induced by TNF-® (Fig. 1, bottom panel; MAC-1 not shown). Normally, LFA-1 and MAC-1 adhere only weakly, but IL-8 or other chemoattractants trigger a conformational change in integrins on the rolling neutrophil, which greatly increases their adhesive capacity. Thereafter, L-selectin is downregulated from the cell surface [8]. The firmly adherent neutrophil may then migrate across the vessel wall towards the peritoneal cavity by a process that is dependent on LFA-1, MAC-1, and ICAM-1. The movement through the vessel wall is known as "diapedesis", the third step in neutrophil recruitment. The fourth, and final, step is the migration of the neutrophil through the submesothelial tissue and to the peritoneal cavity under the influence of chemoattractant cytokines (chemokines) [22]. This directional migration is called "chemotaxis". The most active chemotaxins are the complement split product C5a, bacterial chemotactic peptides (f-MLP) and the CXC chemokines (a cytokine of the IL-8 family, cytokine-induced neutrophil chemoattractant 1 (CINC-1)) [23, 24]. It is thought that C5a is involved in the initial recruitment of neutrophils and that IL-8 is responsible for more prolonged recruitment over a 6–48 hour span [25].

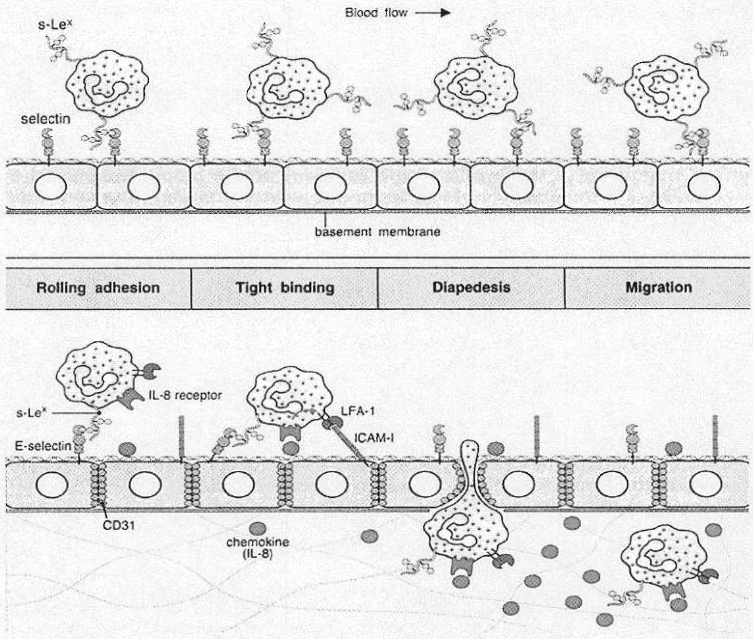

Figure 1. Recruitment of neutrophils, steps 1-4. (Diagram from *Immunobiology. The Immune System in Health and Disease,* Janeway, 4th edition, 1999 [22]).

Mast cells are actively involved in inflammatory and repair processes since they release a wide range of proinflammatory, vasoactive mediators, cytokines, and growth factors in response to activation by immune and non-immune liberators [26]. The migration and the activation of neutrophils during an inflammatory response result from several events. Among these, an important role has been ascribed to the release of chemoattractants by resident cells, and it has been shown that during inflammation, macrophages, mast cells, or lymphocytes control neutrophil influx by release of cytokines, chemokines, or LTB4 [27, 28]. The importance of mast cells in neutrophil recruitment into the peritoneal cavity was confirmed by Ramos et al. [29]. Neutrophil migration induced by IL-8 into the peritoneal cavity was not observed when the peritoneal resident mast cells were depleted, however; nor did Ramos et al. show this IL-8 dependent neutrophil migration to be dependent on LTB4, prostaglandins, or TNF-®[29].

The complement system

Another event resulting in neutrophil activation and migration is the activation of complement system. The complement system is made up of a large number of distinct plasma proteins, which, like the coagulation system, use a cascade of reactions to become activated. The complement system uses three different strategies for recognizing microorganisms and other foreign materials introduced into the body. The *classic pathway* of complement activation is triggered by antibodies bound to antigens on a microbial surface. In this pathway, the complement proteins work together with antibodies to enhance the clearance of antigen. The other two pathways provide for complement activation in the absence of antibody. They are the *alternative pathway* of complement activation, which is triggered directly by constituents of bacterial cell surfaces or foreign surfaces (e.g., biomaterials), and the *lectin-mediated pathway*, which is activated by the binding of a mannose-binding protein present in blood plasma to mannose-containing proteoglycans on the surfaces of bacteria and yeast. All three pathways result in formation of a C3 convertase enzyme (Fig. 2), which cleaves C3 into C3b and C3a fragments. The C3b fragment becomes covalently bound to the pathogen or foreign surfaces. C3b bound to the microbial surfaces can either bind to complement receptors on phagocytic cells, which facilitate phagocytosis of the pathogen, or activate the terminal components of complement, which attack the integrity of the pathogen´s cell membrane. The smaller C3a fragment, together with similar fragments, C4a and C5a, induces inflammation by recruiting inflammatory cells into the area of complement activation.

The complement activation product C5a may be a major chemoattractant for, and activator of, phagocytic cells, but it can also directly activate mast cells [22] and endothelial cells [30]. The latter induce upregulation of P-selectin, thus facilitating adhesion of neutrophils. C5a has been shown to be an important factor stimulating many processes of acute inflammation [31]. The significance of C5a in several inflammatory diseases is demonstrated by the fact that agents that block the action of C5a have also been observed to suppress inflammation in several animal models [30]. However, there has been little direct evidence demonstrating the *in vivo* requirement for C5a in experimental inflammatory models or clinical inflammatory states.

One protein used in experimental models is cobra venom factor (CVF), the complement-activating protein in cobra venom. Cobra venom factor is closely related

to C3, binds mammalian factor B in plasma and, after cleavage of factor B by factor D, produces C3 and C5 convertases that are very stable, with a serum half-life of 7 hours at 37°C [32]. The CVF-Bb convertase consumes all plasma C3. In experimental animals, CVF can wipe out complement activity for between 24 and 72 hours, and with multiple dosing, decomplementation can be maintained for up to 1 week [33]. Cobra venom factor has been shown to be highly effective in many experimental models of complement-mediated pathologies and has provided a useful proof of concept for the notion that complement activation is of relevance in diverse pathologies. Studies using CVF in bacterial and viral infections, in tumor models, and in studies of the immune response have revealed the negative side of anti-complement agent loss of an important player in immune surveillance.

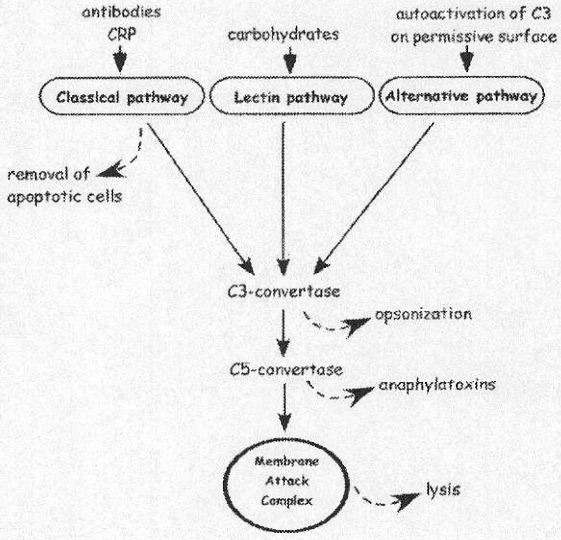

Figure 2. Simplified overview of human complement system. The complement can be activated via three pathways: the classic, alternative, and lectin-mediated pathways.

Complement factors C3a and C5a are known activators of mast cells and phagocytes. The main functions of the phagocytes upon reaching the inflammatory focus are phagocytosis, production of reactive oxygen species (respiratory burst), and reactive nitrogen intermediates (non-oxygen-dependent), needed to eliminate a bacterial or fungal infection. The respiratory burst is an important inflammatory response of the phagocytes. Oxygen radicals are formed both extracellularly and intracellularly inside the phagosome, through a burst of oxygen reduction, which is caused by a

nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex that assembles at the cell membrane and the phagosomal membrane. The production of oxygen radicals is important for the extracellular and intracellular killing of microorganisms [34].

Phagocytosis is initiated by the binding of the microorganisms to the surface of the phagocytic cells, a process that requires some kind of recognition. The microorganism may have been primed with opsonins, IgG and the complement cleavage product C3b being the most important. The opsonins bind to specific receptors such as the polymorphonuclear leukocyte Fc (PMN-Fc), with CR1 supporting the attachment. This stimulates the metabolic activities necessary for killing the microorganism [2], and initiates phagocytosis.

Both free and phagocytosed organisms are removed by transit of peritoneal fluid through the peritoneal lymphatics, principally across the diaphragm [17].

The coagulation system

Just like complement activation, coagulation proceeds through a cascade of reactions, by which inactive factors become enzymatically active following surface contact, or through proteolytic cleavage by other enzymes. The newly activated enzymes in turn activate other, normally inactive precursor molecules. The system is conventionally divided into an intrinsic and an extrinsic pathway that converge on a common pathway, which terminates in thrombin generation (Fig. 3). Thrombin converts soluble plasma fibrinogen to fibrin monomers, which are stabilized by catalyzation of factor XIIIa. Platelets become activated by generated thrombin and are trapped in the fibrin network [35].

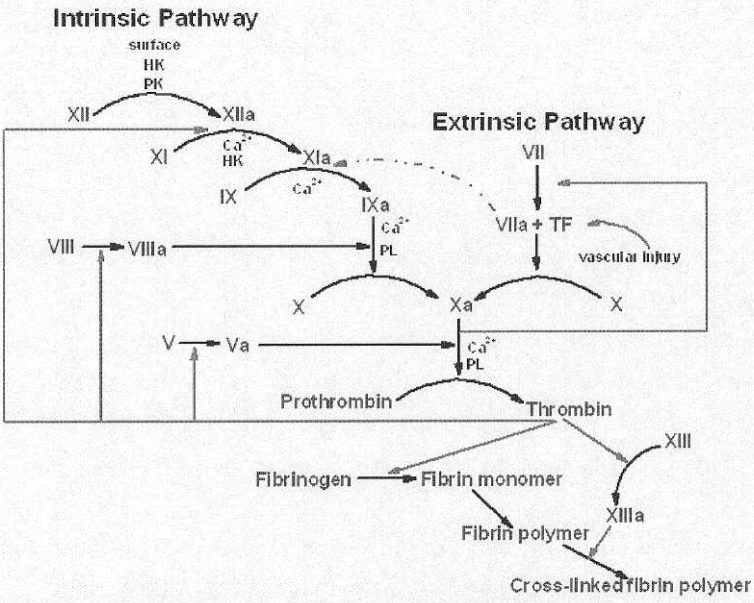

Figure 3. Overview of the coagulation system (Source: M.W.King, 1996).

The coagulation cascade has recently attracted some attention and there are data demonstrating an increased turnover of fibrin in PD patients [36]. Coagulation factor Xa has proinflammatory properties [37, 38]. Thrombin, in addition to its essential role in the process of thrombus formation, plays a pivotal role in the inflammatory immune response and exerts receptor-mediated effects on a number of different cells [39], including mast cells [40], endothelial cells, mesothelial cells [41], monocytes, and neutrophils [26]. The proinflammatory effects of thrombin on cells are probably mediated by membrane-bound protease-activated receptors (PARs) [26]. The thrombin effects include leukocyte adhesion and chemotaxis, cytokine production, and fibroblast proliferation. Thrombin may even induce release of vascular endothelial growth factor (VEGF) [42], thus forming a link between coagulation and angiogenesis.

The effect of instilling continuous ambulatory peritoneal dialysis fluid into the peritoneum

Acute pro-inflammatory effect of peritoneal dialysis fluids

A single i.p. injection of PD fluid causes an inflammatory reaction characterized by the recruitment of neutrophils to the peritoneal cavity [43]. The mechanisms behind this proinflammatory effect largely remain obscure and are a main focus of our studies. The effect may hypothetically be due to certain properties of the PD fluid, such as hyperosmolarity or a low pH, exerting a proinflammatory action [2], possibly mediated through activation of complement, coagulation and mast cells. Mast cells are sensitive to changes in osmolarity [44]. High osmolarity also enhances the production of TNF-α by unstimulated peripheral blood mononuclear cells [45]. However, the mechanisms behind these effects are yet unkown.

Dilution of cells and opsonins

The obvious effect of instilling CAPD intraperitoneally (i.p.) is that both cells and proteins are diluted, initially to very low concentrations. Thereafter, the cell counts and protein concentrations rise [46]. By the end of a clinical PD dwell, the cell concentration is only about 1-10% of normal and the concentration of plasma proteins is only 2-4% of plasma levels [17].

Toxic effects on cells

Degradation of glucose (caramelization) during heat sterilization results in formation of cytotoxic glucose degradation products (GDPs), such as different aldehydes [47], formic acid [17], and 3,4-dideoxyglucosone-3-ene (3,4-DGE) [48]. Glucose degradation products inhibit the *in vivo* respiratory burst response of both peritoneal macrophages [49] and neutrophils [50] and consequently possess anti-inflammatory properties. As well as the toxic effect of the dialysate on the peritoneal phagocytes, the constant removal of large numbers in the dialysate results in a younger population of cells showing fewer signs of maturation [51]. Mesothelial cell production of cytokines is also strongly inhibited [52], as is leukotriene production [53]. Consequently, the peritoneum in CAPD patients acts as an immunocompromised site. Peritoneal dialysis fluids developed more recently contain much lower levels of GDPs due to sterilization in multi-compartment bags.

In most of our experiments, in order to eliminate GDP effects, we used filter-sterilized PD fluids also containing very low GDP levels.

Chronic effects of peritoneal dialysis fluids

Chronic inflammation is characterized by prolonged presence of macrophages, monocytes, and lymphocytes, with proliferation of blood vessels (angiogenesis) and connective tissue, and manifests as persistently high levels of inflammatory mediators [54]. Increased i.p. production of IL-6, IL-8, and IL-1 has been described in peritonitis-free CAPD patients. Even prostaglandin (PGE_2) levels may be increased in the effluents after 1 year of CAPD, which could possibly be linked to mesothelial cells, since isolated macrophages from chronic CAPD patients do not show an enhanced capacity to produce prostaglandins [55].

It is known that long-term CAPD causes structural changes to the peritoneum, at different levels. Documented observations include mesothelial hyperplasia and loss of microvilli, interstitial fibrosis or expansion, thickening of the mesothelial basement membrane, and vascular changes. The structural changes are probably connected to changes in permeability of the peritoneal membrane and in the long run cause ultrafiltration failure, which necessitates transfer of the patient to another treatment modality. No links have, however, been shown between the alternations of the tissue and the clinical outcome [2].

Besides the biological effects of the PD fluids, recurrent peritonitis is the main cause of structural changes to the peritoneal membrane. Consequently, the peritoneal immune system plays an important role in prevention of infectious complications.

Obviously acute infectious peritonitis implies the entry of pathogenic organisms into, and their multiplication within, the peritoneal cavity. In some instances, poor technique, poor training, and poor hygiene are clearly at fault; but in the majority of patients with recurrent peritonitis, there are no records of more frequent prior-technique failure than during periods without peritonitis. The recently developed PD fluids, with lower GDP content, are less toxic to cells of the peritoneal immune system. Despite this, there is no data showing that the new fluids reduce peritonitis frequency or outcome in clinical studies. Attention has therefore turned to host factors, which may determine poor resistance to the microorganisms involved. This in turn has led to studies of normal peritoneal immunology [17].

Additives in peritoneal dialysis fluids

Use of additives in the PD context is a new practice and generally aims to increase the dialysis fluid's biocompatibility. Heparin, a glycosaminoglycan, is the most frequently used drug in PD, and serves the purpose of preventing fibrin formation and catheter obstruction [56]. In a recent cross-over study, 3 months' treatment with low-molecular-weight heparin (LMWH) increased ultrafiltration and reduced glucose transportation in PD patients [57].
Low-molecular-weight heparin is a direct inhibitor of factor Xa and has also demonstrated an ability to inhibit angiogenesis, induced by various agents, in the rat mesentery [58, 59]. Low-molecular-weight heparin has effects on the complement system [60] as well as on the coagulation system, in addition to possible direct effects on VEGF [59] and other growth factors [61]. In animal models, heparins and related compounds have demonstrated the ability to increase ultrafiltration, especially in the presence of i.p. inflammation. It has been assumed that anti-inflammatory actions are responsible for this effect [62, 63].

Another glycosaminoglycan under investigation is hyaluronan (HA), a high-molecular-weight (HMW) linear polysaccharide [64], which is naturally produced in the peritoneal cavity by peritoneal mesothelial cells, fibroblasts, and, to a lesser extent, macrophages. When added to the dialysis fluid, HA has been shown to exert anti-inflammatory and anti-fibrotic actions on the *in vitro* cultured human mesothelial cells (by inhibiting the synthesis of monocyte chemoattractant protein-1 (MCP-1), s-ICAM, vascular endothelial growth factor (VEGF), and fibronectin) and accelerate their growth rate, which may be important for peritoneal healing during PD [65]. However, the clinical effect of HA is under evaluation.

AIMS

The aims of the present thesis were to:

- examine potential interactions between complement factor C5a, the coagulation system, and a member of the IL-8 family, CINC-1, and evaluate the possible contribution of C5a to the acute inflammatory response to PD fluids

- examine the possible role and involvement of mast cells in the acute peritoneal inflammatory response to PD fluids

- investigate the effect of LMWH on peritoneal ultrafiltration, complement, and the coagulation system

- study the effect of heat-sterilized glucose PD fluids *in vivo* on complement activation, CINC-1 levels, neutrophil recruitment, and respiratory burst response during experimental peritonitis.

MATERIALS AND METHODS

Peritoneal dialysis fluids

The laboratory made PD fluid, used in this thesis was composed of the following ingredients: glucose 140 mM, sodium chloride 92 mM, calcium chloride 1.35 mM, magnesium chloride 0.25 mM, and sodium lactate 40 mM. In *paper IV*, pH was adjusted to 5.4 and in papers *I-III* pH was adjusted to 7.2 before sterilization. Sterilization was performed by autoclaving for 1 hour at 120°C (*paper IV*), a method referred to here as "H-PD", or filtering *(paper I-IV)* in 0.2 μm cellulose acetate filters (Nalgene® 0.2 UM SFCA 150 ml Nalgene, NUNC International, New York, NY, USA), a method referred to as "F-PD".

Experimental procedures

1. In vivo

Peritoneal dialysis fluid exposure models

Initially, two different experimental models were used (*papers I and III*) to evaluate the effect of PD on acute inflammatory response in peritoneum. The first model was based on a direct i.p. injection and the second, on a single infusion through an indwelling catheter implanted in the abdominal muscle 6-7 days prior to experiments (*papers I and IV*). By comparing the models, the dependence on known complement-activating mechanisms such as trauma and the presence of a PD catheter could be evaluated. One of the major advantages of the catheter model is that it facilitates taking samples of the i.p. fluid at different time intervals. In addition, the catheter model is more similar to clinical PD than the injection model.

Effects of heparin on ultrafiltration, complement, and coagulation

The effect of LMWH in PD in rats was studied after addition of LMWH to the PD fluids. The animals were exposed to PD fluids during a 2-hour dwell using the catheter and the injection models.

A LMWH preparation with a molecular weight of 5,000 Dalton and a specific activity of 125 U/mg (Fragmin®; Pharmacia Sverige AB, Stockholm, Sweden) was added to 20 ml PD fluid in some animals. Untreated and sham-injected animals were included as controls. In the catheter model, samples of i.p. fluid taken immediately after the PD fluid infusion served as controls and paired comparisons were made with samples taken after 2 hours. Experiments were ended by performing peritoneal lavage. Analyses of the retrieved fluid samples included drained volume, i.p. volume, cell numbers, glucose concentration, complement activation, thrombin production, and *in vitro* chemotactic activity. In some animals, complement and coagulation factors were measured from plasma samples obtained immediately before and after the PD dwell. Additional *in vitro* experiments were performed in order to study the direct blocking effect of LMWH on rat C5a/C5a-desArg.

Evaluation of anti-inflammatory glucose degradation product effects in peritoneal dialysis in experimental peritonitis

In order to investigate the impact of GDPs on leukocyte recruitment to the peritoneal cavity and the capacity of recruited neutrophils to mount a respiratory burst after *in vivo* exposure, two different models of experimental peritonitis were used. Both were based on i.p. fluid injections (20 ml) and dwell times of 2 or 4 hours.

In the first model, peritonitis was induced by zymosan particles (4.4 mg zymosan A #Z-4250; Sigma Chem. Co., St. Louis, MO, USA). The zymosan particles were suspended in 20 ml of either Hanks buffered salt solution (HBSS) or heat-sterilized or filtered PD fluid, and injected i.p. Both unopsonized zymosan particles and particles opsonized for 30 minutes in rat plasma at 37°C were studied. Intraperitoneal complement activation was measured from the concentration of C3a-desArg, and i.p. release of CINC-1 was measured directly from the samples of peritoneal lavage fluid. Neutrophil recruitment was assessed from the cell counts in the peritoneal lavage fluid after a 4-hour dwell and from differential counts made on cytocentrifuge preparations. The respiratory burst of the neutrophils in response to opsonized zymosan was evaluated *in vitro* by chemiluminescence. The effects of PD fluid without zymosan were evaluated in separate experiments.

In the second model, chemical peritonitis was induced by an i.p. injection of HBSS. The PD fluids (heat- or filter-sterilized) were injected i.p. after preexposure for 8

hours to HBSS [49, 50]. After a 2-hour PD fluid dwell, peritoneal lavage was performed. Cells were characterized and counted. The respiratory burst of the neutrophils in response to opsonized zymosan was evaluated *in vitro* by chemiluminescence.

Effects of complement C5 in vivo blockade and the role of CINC-1 in peritoneal dialysis

Using the catheter model, rats were exposed to a single dose of 20 ml of filter-sterilized PD fluid with or without addition of anti-rat C5 antibody 0.1 mg/ml (18A10.62; Alexion Pharmaceuticals, Cheshire, CT, USA). Samples of i.p. fluid, taken immediately after the PD fluid infusion, served as controls, and paired comparisons were made with samples taken after 2 and 4 hours. The dwell fluid was analyzed with regard to activation of the coagulation cascades, neutrophil recruitment, ultrafiltration volume, CINC-1, glucose, and histamine concentrations, and *ex vivo* i.p. chemotactic activity by blocking complement factor C5 and CINC-1.

Functional study of peritoneal mast cells and complement

The catheter model was used to expose the animals to a single dose of 20 ml filter-sterilized fluid. Groups of animals were pretreated either with the mast cell stabilizer doxantrazole (Dox) (3-(1H-tetrazol-5-yl)-9H-thioxanten-9-one 10, 10-dioxide monohydrate) (catalogue #34,640-3; Sigma-Aldrich Co. Ltd., Gillingham, UK), 10 mg/kg body weight (BW), administered intravenously (i.v.), 1 hour prior to PD, or decomplemented with CVF isolated from *Naja melanoleuca* (Venom Supplies Pty. Ltd., Tanunda, South Australia, Australia), 100 μg/kg BW, given i.v. 24 hours prior to exposure to filter-sterilized PD through an indwelling catheter, and compared with untreated controls. Samples of i.p. fluid, taken immediately after the PD fluid infusion, served as controls, and paired comparisons were made with samples taken after 2 and 4 hours. Analyses of the retrieved fluid samples included i.p. volume, cell numbers, and determination of histamine, rat mast cell protease 2 (RMCP-2), and CINC-1 levels.

2. *Ex vivo/in vitro*

Evaluating intraperitoneal chemotactic activity

In *paper I*, human polymorphonuclear granulocytes were used as probes to determine the chemotactic activity of the rat lavage fluid, due to their known cross-reactivity to rat C5a and C5a-desArg [66]. The cells were obtained from whole blood of healthy adult volunteers by density gradient separation, using Percoll® (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the technique described by Braide and Bjursten [67]. The cells were washed and suspended in HBSS, pH 7.20, before being used in the chemotactic assay.

In *paper II*, rat polymorphonuclear granulocytes were used as probes to determine the chemotactic activity of the i.p. fluid. The cells were obtained from whole blood of male Sprague-Dawley rats by density gradient separation using Percoll® (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the technique described by Nannmark et al. [68]. The cells were washed and suspended in phosphate-buffered saline (PBS), pH 7.20, for use in the chemotactic assay.

Chemotaxis was measured in a twelve-well chemotaxis chamber (Neuro Probe, Inc., Gaithersburg, MD, USA). The chamber was equipped with a single polycarbonate filter membrane (25 x 80 mm, pore size 3 μm, thickness 10 μm) and the exposed filter area for each well was 18 mm^2. In *paper I*, the lower compartment of the chamber was filled with 160 μl PD fluid. Each fluid sample was distributed to three different wells for studying chemotaxis, in the presence or absence of anti-C5 antibody, 0.1 mg/ml (18A10.62; Alexion Pharmaceuticals, Cheshire, CT, USA), and chemokinesis. *In paper II*, the lower compartment of the chamber was likewise filled with 160 μl PD fluid. Each fluid sample was then distributed to three different wells for studying chemotaxis, in the presence or absence of anti-rat CINC-1 antibody, 0.1 mg/ml (Lot #AIM014021; R&D Systems, UK), or anti-rat CINC-1 antibody in combination with anti-C5 antibody, 0.1 mg/ml.
The upper compartment was filled with 120 μl HBSS (*paper I*) and PBS (*paper II*) in the chemotaxis experiments and with PD fluid in the chemokinesis experiments. The chemotactic peptide f-Met-Leu-Phe 10^{-8} M in HBSS was used as positive control and HBSS alone was used as negative control in this assay. The chamber and fluids were warmed to 37°C and the wells were loaded with 30 μl of cell suspension (4 x 10^6

granulocytes/ml) to the upper surface of the filter. After incubation for 40 minutes at 37°C in a humidified chamber, the filter was removed and the cells were scraped from the top side of the filter. Remaining cells were then fixed in 4% formaldehyde for 10 minutes before labeling with propidium iodide (2 μg/ml; Molecular Probes, Eugene, OR, USA) for 7 minutes. The filter was mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA) and evaluation was performed within 1 day by fluorescence microscopy in order to quantify neutrophil migration. Chemotaxis was expressed as the number of cells that migrated through the membrane in relation to that in the positive control (i.e., f-Met-Leu-Phe) well.

Blocking of C5a-induced chemotaxis in vitro

The chemotaxis assay employed to determine *in vitro* chemotactic activity of PD fluids was also used to study the blocking effects of LMWH and the monoclonal antibody (MAb) 18A10.62 on rat C5a/C5a-desArg in *paper I*. Zymosan-activated serum used as the source of C5a was prepared by incubating rat serum diluted 1:1 in HBSS for 1 hour with zymosan 20 mg/ml. After removing the zymosan by centrifugation, the serum was stored frozen until used. The blocking agents were added to both compartments and chemotaxis was compared between blocked and unblocked chambers. The LMWH level was studied at concentrations of 6.25 and 3.12 U/ml, the higher concentration corresponding to that used in PD fluid *in vivo*. The antibody 18A10.62 was studied at a concentration of 0.1 mg/ml. Chemotaxis was expressed as the number of migrated cells in relation to the positive control (i.e., f-Met-Leu-Phe) well.

Animals

Anesthesia

The study protocol was approved by the Göteborg Ethics Committee. Male Sprague-Dawley rats weighing between 250 g and 400 g were used in all experiments. General anesthesia was induced and maintained by inhalation of Isofluran Baxter® (Baxter Medical AB, Kista, Sweden) in room air *(papers I-III)*.

In *paper IV*, general anesthesia was induced by an intramuscular (i.m.) injection of pentobarbital sodium (1.5 mg/100 g BW) and diazepam (0.25 mg/100 g BW). During catheter implantation, the duration of anesthesia was approximately 20 minutes. During injections and infusions, the total duration of anesthesia was <10 minutes and the animals were awake during the rest of the dwell. At the end of the dwell, the animals were sacrificed under general anesthesia.

The catheter model

A 7 French silicone catheter (Renasil® SIL080; Braintree Scientific Inc., Braintree, MA, USA) was implanted under sterile conditions and general anesthesia. A midline incision was made through the abdominal skin and a hole was pierced through the linea alba with a 3 mm diameter needle, taking care not to cause any bleeding. After inserting 2.5 cm of the tip through the hole, the catheter was sutured to the superficial abdominal muscle fascia and the rest of the catheter was tunneled subcutaneously (s.c.) to the neck region and mobilized through the skin. After injecting 5 ml of saline, the catheter was closed with a stainless clip. The wounds were closed with wound clips.

At 6-7 days after the catheter implantation, the animals were anesthetized and given a single 20 ml infusion of filter-sterilized PD fluid, with or without additive. Texas Red-labeled albumin was included as volume marker. A 1 ml fluid sample was drawn through the catheter and the animals were allowed to wake up. Two hours later (*paper I*) or 2 and 4 hours later (*papers II and III*), the animals were anesthetized again and a new 1 ml fluid sample was drawn through the catheter. Before samples were taken, the animals were gently turned and their abdomens massaged in order to mix the i.p. fluid. The samples from the catheter at 0 and 2 (*paper I*) and 0, 2, and 4 hours (*paper III*) were used for the analyses. In order to determine the rate of lymphatic fluid reabsorption from the i.p. space, a final fluid sample was taken after infusion of an additional 10 ml volume of PD fluid without labeled albumin. The animals were then sacrificed.

Wash-out procedure

In *paper III*, a group of animals were treated by an initial wash-out of the peritoneal cavity by immediately draining 12 – 15 ml of the infused PD fluid and replacing it

with the same volume of fresh fluid. The dwell and sampling was performed as with the other groups of animals.

The injection model

A single dose of 20 ml of heat- or filter-sterilized PD fluid, with or without additive, was injected into the abdominal cavity by a short needle. At the end of the 2-hour dwell, peritoneal lavage was performed by injecting 20 ml of PBS supplemented with 20 U/ml unfractionated heparin, gently shaking the animal and emptying the fluid through an abdominal midline incision. The animals were then sacrificed. The weight of the lavage fluid was determined and an EDTA-treated sample was retained for analysis of cytology and complement and coagulation factors.

Analyses, measurements, and evaluations

Cytological evaluation

The samples were centrifuged at 400 g for 10 minutes in order to separate the blood cells, which were resuspended in a small volume of HBSS and counted. Cytospin preparations were made and evaluated cytologically (x 100 magnification) after staining in hematoxylin-eosin (H & E). At least 100 random cells were counted per slide and classified as basophils, eosinophils, neutrophils, or macrophages/monocytes. Cells that appeared to be damaged were excluded from the evaluation.

Measurements of glucose, histamine, and urea concentrations

The cell-free samples (*papers I and II*) were diluted 1:5 with lactate buffer at pH 5.5. After calibration for this dilution, glucose concentrations were measured with Ascensia Contour and Ascensia Microfill sticks (Bayer HealthCare, Bayer AB, Division Diagnostika, Göteborg, Sweden) based on pyrroloquinoline quinone (PQQ) glucose dehydrogenase and potassium ferricyanide.

Histamine concentrations (*paper II*) were determined fluorometrically after reaction with o-phtalaldehyde according to the method described by Fredholm and Haegermark [69]. Urea concentrations (*paper I*) were determined from cell-free

samples, diluted 1:4 in distilled water, by applying a modification of the Sigma assay kit [70].

Analyses of complement, coagulation, and cytokine-induced neutrophil chemoattractant 1 and rat mast cell protease 2 concentration

In *paper I*, sodium EDTA, at a final concentration of 10 mM, was added to one portion of the peritoneal lavage fluid (injected animals) or present in the sampling device connected to the catheter or the blood vessel. Blood samples were spun to remove the blood cells. In *paper II*, sodium EDTA, at a final concentration of 10 mM, was added to all samples (taken at 0, 2, and 4 hours).

Complement activation (*papers I and IV*) was determined from the concentration of C3a-desArg measured by enzyme-linked immunosorbent assay (ELISA) (rat C3a-desArg ELISA #CL89160K; Cedarlane Laboratories Ltd., Hornby, Ontario, Canada), according to the manufacturers' instructions, in samples diluted 1:20.

Coagulation was determined from measurements of the thrombin-antithrombin (TAT) complex (Enzygnost® TAT micro #OWMG 15; Dade Behring, Marburg GmbH, Marburg, Germany) (*papers I and II*). C3a-desArg and TAT levels were expressed as calculated total i.p. quantities (*papers I and II*) or, in the case of plasma samples, as concentrations (*paper I*).

Cytokine-induced neutrophil chemoattractant 1 levels in *papers II-IV* were determined from the supernatants of i.p. fluid samples (Quantikine® Rat CINC-1 Immunoassay, #RCN100; R&D Systems Europe, Ltd., UK).

Rat mast cell protease 2 concentrations, in *paper III*, were determined from supernatants and plasma samples diluted 1:1 (RMCP-2 ELISA Kit, Cat.# MS-RM2; Moredun Scientific Limited, Edinburgh, Scotland, UK) according to the manufacturers' instructions.

Measurements of respiratory burst

Luminol (5-amino-2.3-dihydro-1.4-phatalzinedione, 5.5 mg/ml, in DMSO; Sigma Chem. Co., St. Louis, MO, USA)-amplified chemiluminescence was used in *paper IV* to measure the respiratory burst of the peritoneal leukocytes *in vitro*. The respiratory

burst was measured in response to zymosan opsonized in rat serum (30 minutes at 37°C).

A sample of the peritoneal cell suspension was diluted in order to obtain a cell count of 4.0 x 10^6 leukocytes/ml. An amount of 100 µl of the cell suspension was mixed with 100 µl luminol, diluted 1:20 in HBSS, and 100 µl of either opsonized zymosan (12.5 mg/ml) or HBSS used as a control. The samples were immediately loaded into a luminometer (125l luminometer; LKB Wallac Oy, Turku, Finland) controlled by a desktop computer. Measurements were performed every 30 seconds for 20 minutes and the mean values in mV were used in the evaluation.

Determination of intraperitoneal fluid volume

Fluorescently labeled albumin was used as a volume marker, according to a recently developed technique described by Bazargani et al. [71]. In brief, 100 µg of Texas Red-labeled bovine serum albumin (TR-BSA) (Molecular Probes, Eugene, OR, USA) and 1 mg of unlabeled BSA were added to the PD fluid and a 1 ml sample was retrieved before i.p. injection. Intraperitoneal volumes were determined, between different cell-free samples, by spectrophotometric analysis (570 nm excitation and 615 nm emission). An intentional dilution of 10 ml PD fluid without TR-BSA, performed at the end of the experiment, enabled measurement of the true i.p. fluid volume at the time and was used to calculate the total fluid reabsorption during the 4-hour dwell.

Statistical analysis

Throughout the analysis of data, non-parametric statistics were used. However, in the presentation, measured values are expressed as means ± standard error of the mean (SEM). The Wilcoxon-Mann-Whitney test (Mann-Whitney's U-test in *paper IV*) and Wilcoxon's signed-ranks test were used to evaluate differences between independent and paired samples, respectively, and the Spearman rank-order correlation coefficient was used to evaluate correlations. Compensation for mass significances was performed using the sequentially rejective Bonferroni correction. The chosen level of significance was $p \leq 0.05$.

RESULTS

Effect of low-molecular-weight heparin on peritoneal dialysis in animals (paper I)

Initially, we focused on the effect of LMWH on ultrafiltration, coagulation, and complement activation during a single PD dwell. In this paper, we evaluated the effects of LMWH supplementation on ultrafiltration, coagulation, and complement activation during a single PD dwell. The injection and the catheter models were studied and compared.

The i.p. volumes obtained from measurements of Texas Red® albumin after PD fluid exposure increased in both models when LMWH was added to the PD fluid (Fig. 4 [Fig. 1 in the paper]). The difference was significant in both the injection model and the catheter model.

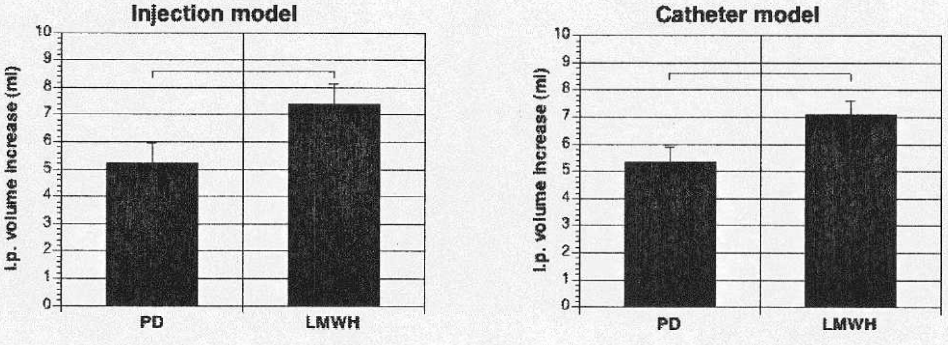

Figure 4. Intraperitoneal (i.p.) volume increases, following dilution of Texas Red-labeled albumin, 2 hours after an i.p. injection (**left**) or a catheter infusion (**right**) of 20 ml of peritoneal dialysis (PD) fluid. Addition of 125 U low-molecular-weight heparin (LMWH) to the PD fluid significantly increased the drained volumes, indicating improved ultrafiltration. Brackets indicate statistically significant differences.

Without supplementation with LMWH, an i.p. injection of 20 ml PD fluid induced neutrophil recruitment (Fig. 5; Table 1) and activation of the complement (Fig. 6) and coagulation (Fig. 7) cascades.

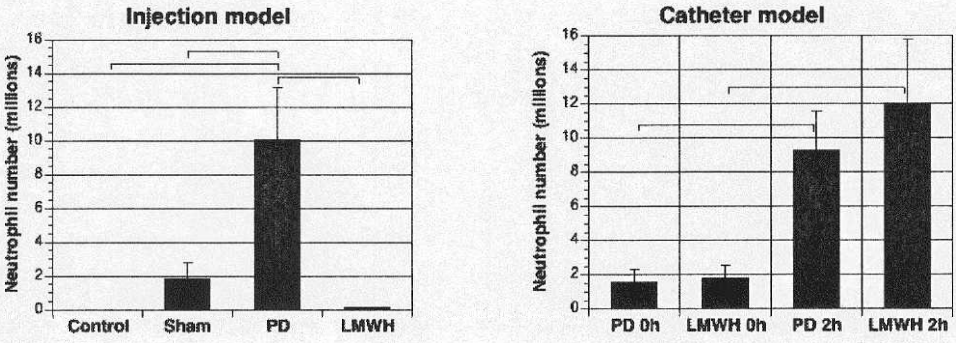

Figure 5. Peritoneal neutrophil recruitment, measured as intraperitoneal (i.p.) neutrophil numbers (in millions), 2 hours after an i.p. injection (**left**) or a catheter infusion (**right**) of 20 ml of peritoneal dialysis (PD) fluid. In the injected animals, the neutrophil numbers increased significantly compared with untreated (control) or punctured (sham) animals in the absence of low-molecular-weight heparin (LMWH). Addition of 125 U LMWH to the PD fluid significantly inhibited neutrophil recruitment. In the catheterized animals, the neutrophil numbers increased significantly over the 2-hour PD dwell in the presence or absence of LMWH. Brackets indicate statistically significant differences.

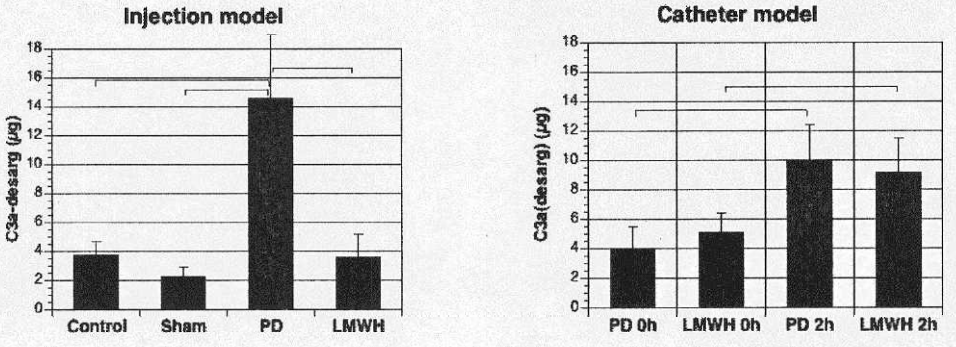

Figure 6. Peritoneal complement activation, measured as intraperitoneal (i.p.) C3a-desArg quantity, 2 hours after an i.p. injection (**left**) or a catheter infusion (**right**) of 20 ml of peritoneal dialysis (PD) fluid. In the injected animals, the amount of C3a-desArg increased significantly compared with untreated (control) or punctured (sham) animals in the absence of low-molecular-weight heparin (LMWH). Addition of 125 U LMWH to the PD fluid significantly reduced the C3a-desArg level. In the catheterized animals, the C3a-desArg quantity increased significantly over the 2-hour PD dwell in the presence or absence of LMWH. Brackets indicate statistically significant differences.

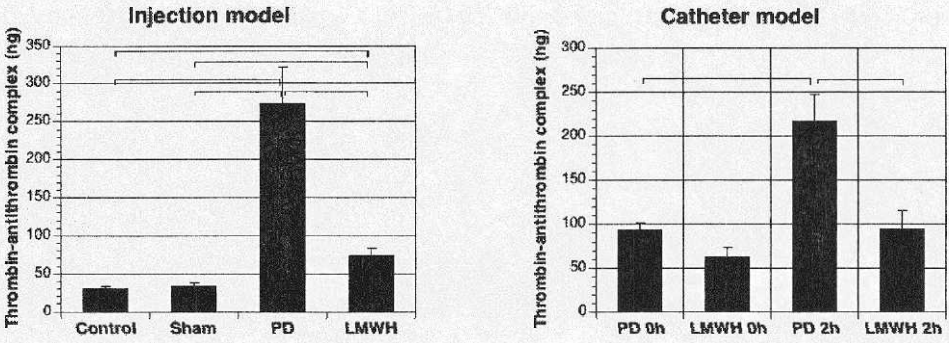

Figure 7. Peritoneal thrombin formation, measured as intraperitoneal (i.p.) thrombin-antithrombin (TAT) quantity, 2 hours after an i.p. injection (**left**) or a catheter infusion (**right**) of 20 ml peritoneal dialysis (PD) fluid. In the injected animals, the amount of TAT increased significantly compared with untreated (control) or punctured (sham) animals in the presence or absence of low-molecular-weight heparin (LMWH). In the catheterized animals, the quantity of TAT increased significantly over the 2-hour PD dwell in the absence of LMWH. Addition of 125 U LMWH to the PD fluid significantly inhibited the thrombin formation in both models. Brackets indicate statistically significant differences.

Table 1

Model	**Treatment**	*Cell numbers, in millions*			
		Mononuclear cells	**Neutrophils**	**Eosinophils**	**Basophils**
Injection	**PD**	7.66±1.69	10.03±3.14	2.22±0.51	1.37±0.36
	LMWH	12.90±0.88	0.10±0.05	1.98±0.32	1.94±0.48
	Sham	10.95±1.29	1.61±0.84	1.94±0.48	1.26±0.29
	Control	11.57±1.25	0.00±0.00	2.07±0.46	0.62±0.20
Catheter	**PD, 0 h**	4.71±1.51	1.67±0.87	3.32±1.08	0.02±0.02
	LMWH, 0 h	4.87±2.15	1.85±0.69	5.87±0.90	0.09±0.06
	PD, 2 h	11.33±5.03	9.27±2.35	7.08±2.13	0.00±0.00
	LMWH, 2 h	13.69±8.38	11.95±3.88	13.06±3.17	0.00±0.00

Cell numbers, in millions, of different intraperitoneal (i.p.) leukocyte populations obtained from control animals, 2 hours after an i.p. injection and before (0 hours) and after (2 hours) a catheter infusion of 20 ml of peritoneal dialysis (PD) fluid. In untreated and sham-injected animals, the mononuclear cells dominated. In the catheterized animals, eosinophils and mononuclear cells dominated at 0 hours. Exposure to PD fluid increased the neutrophil fractions, but in the injected animals, this increase was inhibited by low-molecular-weight heparin (LMWH) supplementation.

Addition of LMWH significantly reduced cogulation (the amount of TAT present) after PD fluid exposure in both models (Fig. 7). In the injection model, the neutrophil numbers and complement activation (C3a-desArg quantity) after PD fluid exposure were also reduced by LMWH (Figs 5 and 6; Table 1). In the catheter model, there were no such effects. *In vitro* chemotactic activity was significantly increased after exposure to PD fluid in both models (Fig. 8). *In vitro* chemotactic activity of the lavage fluid was significantly inhibited by the addition of LMWH in the injected animals (Fig. 8). In the catheterized animals, there was a non-significant ($p<0.076$) tendency towards chemotaxis inhibition by LMWH.

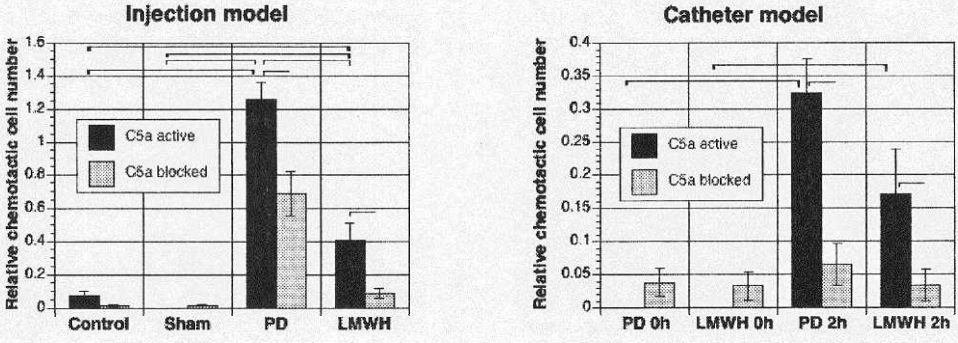

Figure 8. *In vitro* chemotactic activity (relative number of migrated neutrophils in a chemotaxis chamber) of intraperitoneal (i.p.) fluid obtained 2 hours after an i.p. injection (**left**) or a catheter infusion (**right**) of 20 ml of peritoneal dialysis (PD) fluid. In the injected animals, the chemotactic activity significantly increased compared with untreated (control) or punctured (sham) animals. In the catheterized animals, the chemotactic activity increased significantly over the 2-hour dwell. Addition of 125 U low-molecular-weight heparin (LMWH) to the PD fluid significantly reduced the chemotactic activity in the injected animals. *Ex vivo* blocking of C5a with antibodies in the PD fluid samples significantly reduced the number of migrating neutrophils. Brackets indicate statistically significant differences.

Blocking the PD fluid samples for C5a *ex vivo* led to a substantial and significant reduction in the number of chemotactic cells, indicating that a considerable part of the chemotactic activity was due to the presence of C5a. After blocking of C5a, the remaining chemotactic activity was, however, still significantly elevated in the injection model.

The direct inhibitory effect of LMWH on C5a/C5a-desArg was studied *in vitro* in the chemotaxis assay using rat zymosan-activated serum as C5a source. At the concentration used in the *in vivo* experiments (6.25 U/ml), LMWH completely inhibited chemotaxis of human neutrophils (Fig. 9). At the lower concentration, 3.12 U/ml inhibition was weaker. The concentration of LMWH was significantly negatively correlated to chemotaxis (correlation coefficient rho = -0.93).

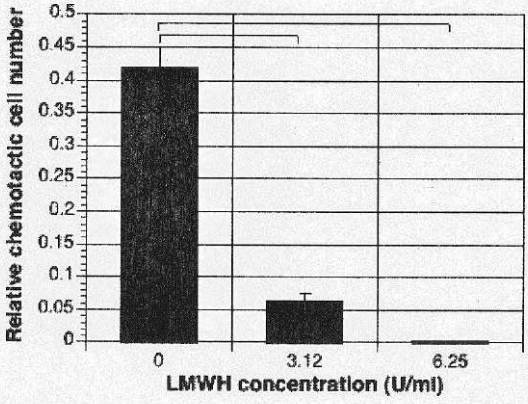

Figure 9. *In vitro* chemotactic activity (relative number of migrated neutrophils in a chemotaxis chamber) of zymosan-activated rat serum supplemented with LMWH at two concentrations. At the higher concentration of 6.25 U/ml, which corresponded to the concentration used *in vivo*, inhibition of chemotaxis was total, indicating that LMWH blocks C5a and C5a-desArg, the chemotaxins present in zymosan-activated serum. Brackets indicate statistically significant differences.

Role of complement factor C5a andCINC- 1 in peritoneal dialysis (paper II)

In the second study (*paper II*), we took our investigation a step further, to evaluate the possible contribution of complement factor C5a and the potential interactions between C5a, the coagulation system, and a member of the IL-8 family, CINC-1. Ultrafiltration, glucose transport, neutrophil recruitment, and the levels of several mediators of inflammation and coagulation were studied in the catheter model. Monoclonal antibodies were used to block C5 *in vivo*.

The levels of CINC-1 (Fig. 10), TAT (Fig. 11), and histamine (Fig. 12) and the neutrophil numbers (Fig. 13) increased significantly during the PD dwell. The C5

blockade significantly reduced the initial levels of TAT and increased the ultrafiltration volumes at 2 hours (Fig. 14). Glucose concentrations were significantly positively correlated to ultrafiltration volumes at two hours and showed a tendency to increase under C5 blockade (Fig. 15).

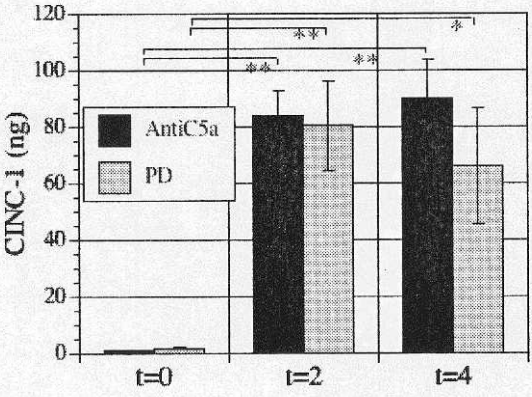

Figure 10. Cytokine-induced neutrophil chemoattractant 1 (CINC-1) increased significantly during the dwell in both groups of animals (**p≤0.01; *p≤0.05). Compared with initial quantities, values measured at 2 and 4 hours were significantly higher.

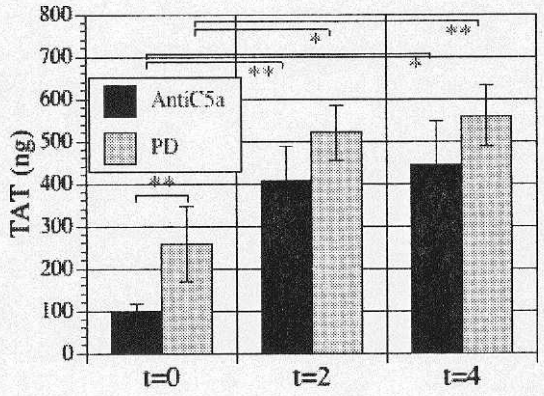

Figure 11. Levels of the thrombin-antithrombin (TAT) complex increased over time in both groups, indicating that activation of the coagulation system was induced by the PD dwell (**p≤0.01; *p≤0.05). In the samples taken at 0 hours, TAT levels were significantly lower in the presence of C5 blockade (p<0.01).

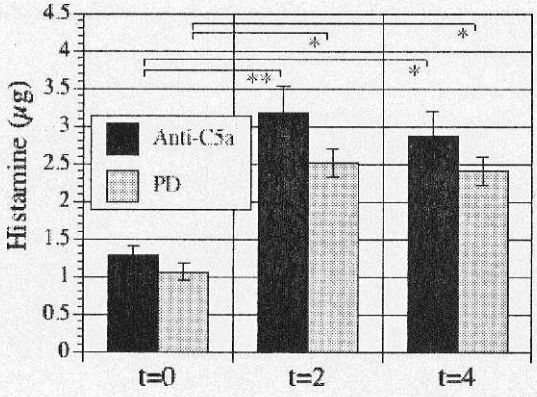

Figure 12. Intraperitoneal (i.p.) histamine levels increased in both groups after initiation of the dwell. Histamine quantities at 2 and 4 hours were significantly higher than initially with and without C5a blockade (**p≤0.01; *p≤0.05).

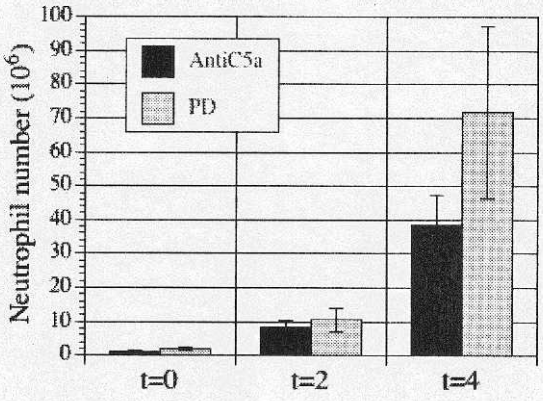

Figure 13. Neutrophil numbers increased over time during the dwell in both groups of animals, although there was no significant difference between the groups at any time point.

In order to detect the factors behind the chemotactic activity of the PD fluid samples, CINC-1 and C5 were blocked with MAbs *ex vivo* before measuring chemotactic activity. After blockade, chemotactic activity was below detectable limits at all three time points in both groups (data not shown), demonstrating that either C5a or CINC-1 mediated all chemotactic activity in the samples. In order to quantify the C5a-dependent chemotactic activity, CINC-1 was blocked *ex vivo* before measuring chemotaxis. As expected, the remainder of chemotactic activity in animals treated with anti-C5 *in vivo* was virtually abolished at all time points (Fig. 16, right panel). In

the untreated group, *ex vivo* blockade of CINC-1 resulted in a progressive increase in C5a-dependent chemotaxis over time (Fig. 16, left panel).

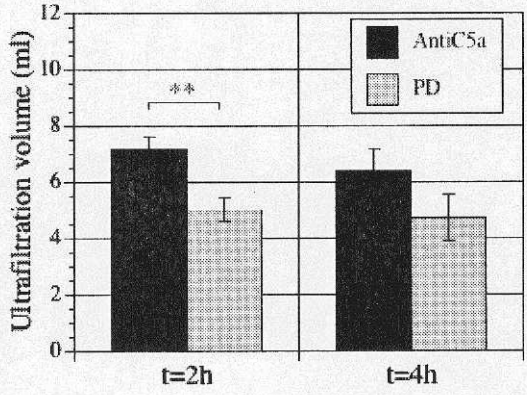

Figure 14. The ultrafiltration volumes at 2 hours, as measured by Texas Red® albumin dilution, increased significantly (** = $p \leq 0.01$) in the presence of C5 blockade. After four hours the difference was insignificant (p=0.12).

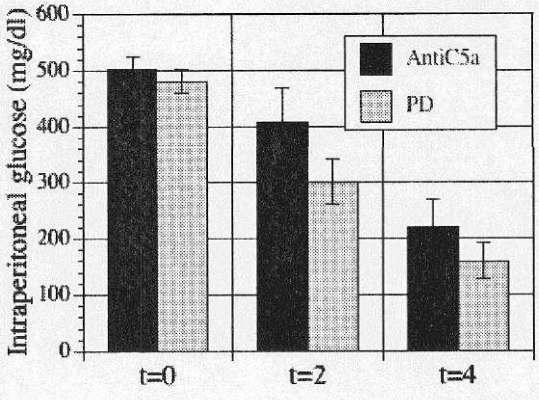

Figure 15. Glucose concentrations were not significantly affected by the C5 blockade, but there was a non-significant tendency towards an increase in total intraperitoneal (i.p.) glucose retention in the anti-C5-treated animals at 2 and 4 hours (p=0.08 and p=0.13, respectively).

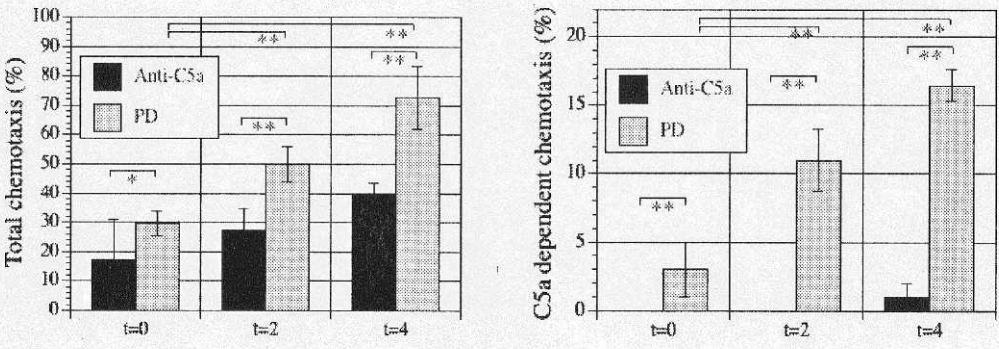

Figure 16. **(Left)** In the animals not blocked for C5 (PD), the total chemotactic activity *in vivo* increased over time and was significantly reduced by the C5 blockade at all time points (**p≤0.01; *p≤0.05).**(Right)** When blocking the samples for cytokine-induced neutrophil chemoattractant 1 (CINC-1) *ex vivo*, only the C5a-dependent chemotactic activity could be specifically measured. As expected, this activity was virtually absent at all time points in anti-C5-treated animals. In the untreated group (PD), C5a-dependent chemotaxis increased progressively over time and was significantly higher than in the C5-blocked animals at 2 and 4 hours (**p≤0.01).

The possible role and involvement of mast cells in the acute peritoneal inflammatory response to peritoneal dialysis fluids (paper III)

The third study (*paper III*) was performed to investigate the role of resident peritoneal mast cells on neutrophil recruitment, and examine whether mast cell activation in PD is secondary to complement activation. The catheter model was combined with the actions of functionally stabilizing the peritoneal mast cells with Dox, a stabilizer of mucosal and connective tissue mast cells, and consuming complement factor C3 by CVF. Untreated animals served as controls.

Exposure to PD fluid invariably led to recruitment of neutrophils (Fig. 17) and release of CINC-1 (Fig. 18) into the peritoneal cavity. The CINC-1 levels increased approximately 20 times in the control group. Treatment with CVF reduced those effects insofar as the CINC-1 increase was only significant at 4 hours ($p<0.05$) and the neutrophil recruitment was only significant at 2 hours ($p<0.05$) following CVF treatment. Intraperitoneal histamine concentration changed little during the dwell, but a significant increase ($p<0.05$) was seen in all groups from 0 hours to 2 hours. At 4 hours, the level remained elevated (Fig. 19).

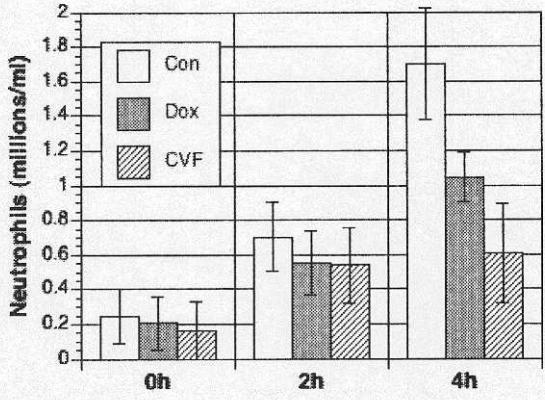

Figure 17. Neutrophil counts in PD fluid samples at 0, 2, and 4 hours of dwell time. Neutrophil counts were significantly higher at 2 hours ($p<0.01$) and 4 hours ($p<0.01$) compared with initial values in the control group (Con). Comparison of neutrophil levels at 2 and 4 hours revealed significant differences, at 4 hours, for doxantrazole (Dox) and cobra venom factor (CVF). Data are given in millions/ml and presented as means ± standard error of the mean (SEM). Each bar represents six to eight animals.

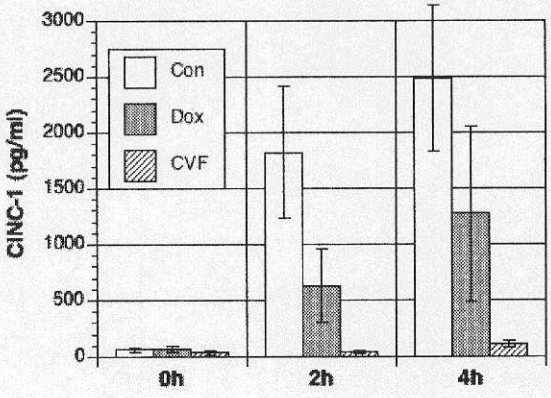

Figure 18. Cytokine-induced neutrophil chemoattractant 1 (CINC-1) concentrations in peritoneal dialysis (PD) fluid samples at 0, 2, and 4 hours of dwell time. The CINC-1 levels increased significantly, approximately 20 times in the control group (Con). Doxantrazole (Dox) did not significantly block the CINC-1 release. In cobra venom factor (CVF)-treated animals, the CINC-1 levels were significantly lower compared with controls at 2 and 4 hours ($p<0.01$). Values are given in pg/ml. Data are presented as means ± standard error of the mean (SEM). Each bar represents six to eight animals.

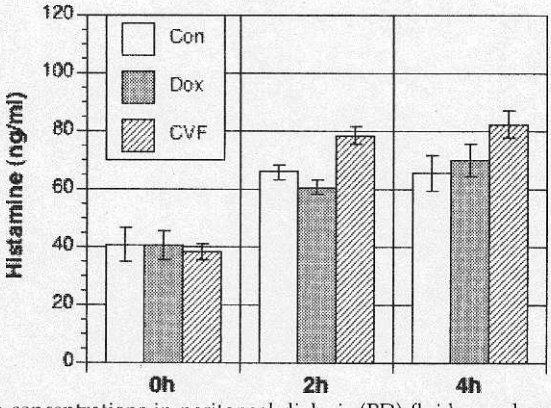

Figure 19. Histamine concentrations in peritoneal dialysis (PD) fluid samples at 0, 2, and 4 hours of dwell time. Intraperitoneal (i.p.) histamine concentrations changed little during the dwell, but there was a significant ($p<0.05$), approximately twofold, increase from time 0 to 2 hours in all groups. The levels remained elevated at 4 hours. Con = control; Dox = doxantrazole; CVF = cobra venom factor. Data, in ng/ml, are presented as means ± standard error of the mean (SEM) and each bar represents six to eight animals.

During the dwell, RMCP-2, a marker of mucosal mast cells, increased slightly but significantly ($p<0.05$) in all groups (Fig. 20).

Wash-out of the peritoneal cavity in the beginning of the dwell did neither reduce neutrophil numbers nor CINC-1 levels significantly at 2 or 4 hours.

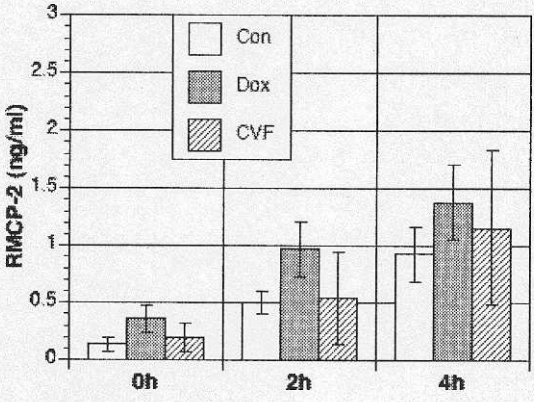

Figure 20. Rat mast cell protease 2 (RMCP-2) concentrations in peritoneal dialysis (PD) fluid samples at 0, 2, and 4 hours of dwell time. Concentrations increased slightly but significantly ($p<0.05$) in all groups during the dwell. Cobra venom factor (CVF) did not have a significant effect on the RMCP-2 levels. Doxantrazole (Dox) significantly ($p<0.05$) increased the RMCP-2 concentration at 2 hours. Con = control. Values are given in ng/ml. Data are presented as means ± standard error of the mean (SEM) and each bar represents between six and eight animals.

CVF significantly increased the net ultrafiltration and the gross ultrafiltration (Fig. 21). Fluid reabsorption rates did not differ between the groups and were 30.7±5.6 μl/min, 24.0±1.1 μl/min and 31.6±5.2 μl/min for controls, doxantrazole and CVF treated animals respectively.

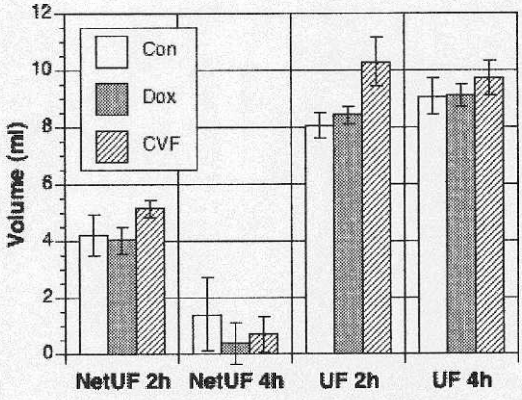

Figure 21. Ultrafiltration volumes excluding (NetUF) and including (UF) lymphatic fluid reabsorption at 0, 2 and 4 hours of dwell time. Decomplementation with cobra venom factor significantly increased ultrafiltration at 2 hours. Con = control; Dox = doxantrazole. Data are means ± SEM in ml and each bar represents 6-8 animals.

Effect of heat-sterilized peritoneal dialysis fluids on complement activation, CINC-1 release, and neutrophil recruitment (paper IV)

The aim of the last study (*paper IV*) was to compare the acute *in vivo* effects of GDPs on neutrophil recruitment, CINC-1 production, and respiratory burst response in relation to different mechanisms of inflammatory activation in the injection model. Experimental peritonitis was induced by zymosan, a known activator of the complement system, allowing a correlation of complement activation, CINC-1 release, and neutrophil recruitment.

Opsonized zymosan caused an inhibition of ultrafiltration, reducing the drained i.p. fluid volume to the level of the isotonic HBSS (Fig. 22). Addition of zymosan to the PD fluid also led to complement activation (Fig. 23), release of CINC-1 (Fig. 24), and recruitment of neutrophils (Fig. 25) to the peritoneal cavity. Heat-sterilized PD fluid significantly inhibited CINC-1 release and the recruitment of neutrophils in this case. When unopsonized zymosan was used, however, neither the neutrophil recruitment nor the CINC-1 release was affected by the type of PD fluid used. In either case, complement activation was unaffected by the choice of PD fluid. Neutrophils recruited after a single injection of HBSS and exposed i.p. to heat-sterilized PD fluid showed a significantly impaired respiratory burst response (Fig. 26).

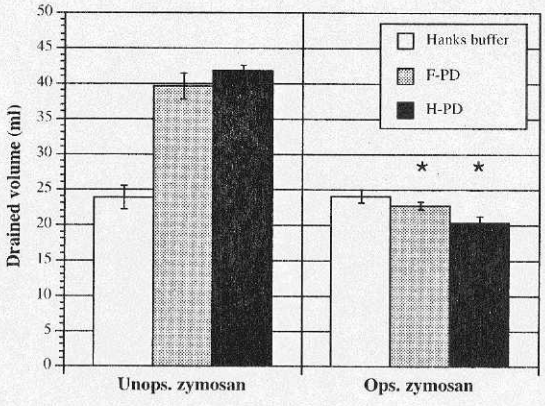

Figure 22. The drained volumes of peritoneal effluents from rats after intraperitoneal (i.p.) exposure to either Hanks buffered salt solution (HBSS) or filter-sterilized (F-PD) or heat-sterilized peritoneal dialysis (H-PD) fluid, with or without addition of zymosan (opsonized or unopsonized). Values, in ml, are presented as means ± standard error of the mean (SEM) and each bar represents five to seven animals. Addition of opsonized zymosan significantly reduced the drained volumes of PD fluids down to the level obtained with HBSS. Asterisks indicate statistically significant differences ($p<0.05$) between opsonized and unopsonized zymosan.

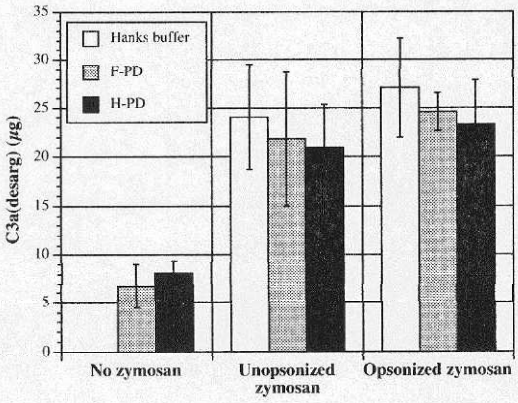

Figure 23. The measured complement activation in peritoneal effluents from rats after intraperitoneal (i.p.) exposure to either Hanks buffered salt solution (HBSS) or filter-sterilized (F-PD) or heat-sterilized peritoneal dialysis (H-PD) fluid, with or without addition of zymosan (opsonized or unopsonized). Values are given in μg. Data are presented as means ± standard error of the mean (SEM) and each bar represents between five and eight animals. Addition of zymosan (opsonized or unopsonized) significantly increased the measured quantities of C3a-desArg. There were no significant differences between treatment with F-PD and treatment with H-PD.

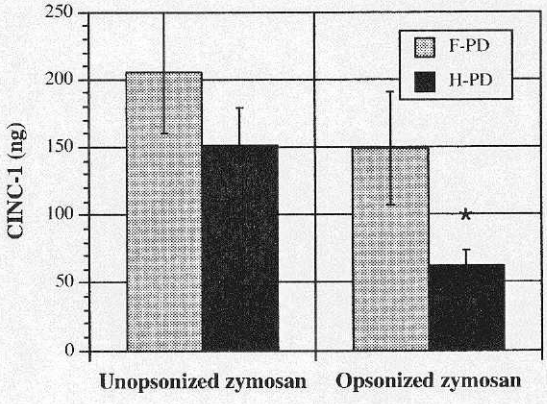

Figure 24. The measured cytokine-induced neutrophil chemoattractant 1 (CINC-1) release in peritoneal effluents from rats after intraperitoneal (i.p.) exposure to opsonized or unopsonized zymosan in either filter-sterilized (F-PD) or heat-sterilized peritoneal dialysis (H-PD) fluid. The H-PD fluid significantly reduced the CINC-1 release induced by opsonized zymosan. The response to unopsonized zymosan did not differ significantly between the two types of PD fluids.

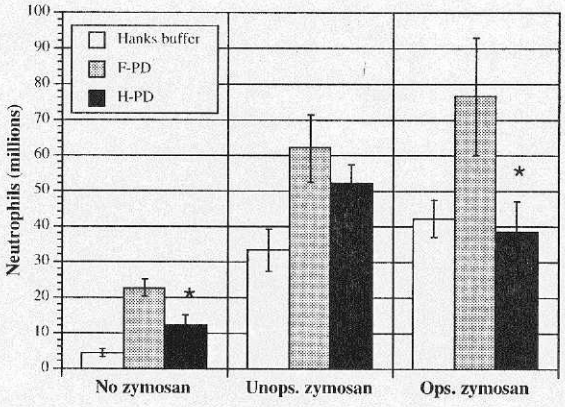

Figure 25. The neutrophil counts in peritoneal effluents from rats after intraperitoneal (i.p.) exposure to either Hanks buffered salt solution (HBSS) or filter-sterilized (F-PD) or heat-sterilized peritoneal dialysis (H-PD) fluid mixed with either opsonized or unopsonized zymosan. Figures are given in millions. Data are presented as means ± standard error of the mean (SEM) and each bar represents five to eight animals. The asterisks indicate a statistically significant difference ($p<0.05$) between F-PD and H-PD fluids. Data on the same fluids without addition of zymosan (No zymosan) are included from a previous study [49].

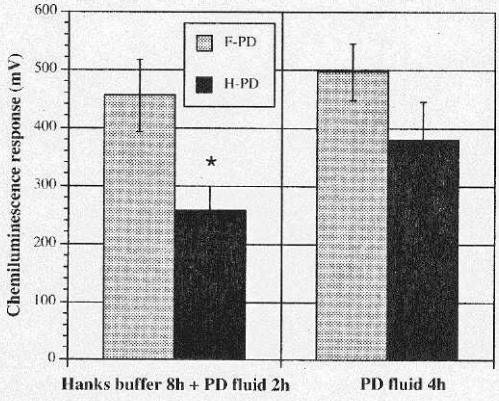

Figure 26. The chemiluminescence in response *in vitro* to neutrophils exposed *in vivo* to either filter-sterilized peritoneal dialysis (PD) fluid (F-PD) or heat-sterilized PD fluid (H-PD), 8 hours after an intraperitoneal (i.p.) challenge with Hanks buffered salt solution (HBSS). Data are presented as means ± standard error of the mean (SEM) and each bar represents six to eight animals. Values are given in mV. Asterisks indicate statistically significant differences ($p<0.05$). Data from a previous study on neutrophils from PD effluents without preexposure to HBSS (PD fluid at 4 hours) are included [49].

DISCUSSION

Inflammatory response in experimental peritoneal dialysis

This work was initiated to investigate the regulatory mechanisms behind the acute inflammatory response, in the peritoneal cavity, to PD. In both models (catheter and injection), exposure to PD fluid by i.p. injection or by a resident catheter led to rapid recruitment of neutrophils and increases in C3a-desArg, TAT and CINC-1 levels, detectable 2 hours later. Those changes were sustained or amplified at 4 hours.

The i.p. baseline quantities of C3a-desArg, TAT and CINC-1 corresponded to those present in 2–3 ml of plasma (*paper I*), indicating that before the PD dwell, there was a steady state between plasma and the i.p. fluid volume (2-3 ml). The rise in C3a-desArg, TAT and CINC-1, seen during the PD dwell, was substantial and by far exceeded the contribution expected from exchange between plasma and the i.p. space. According to preliminary measurements on ^{125}I-labeled albumin, the clearance from plasma to the peritoneum of these proteins should amount to <1 ml during a 2-hour dwell in the present models. Consequently, at least 80% of the observed increases in C3a-desArg and TAT and an even larger fraction of the CINC-1 increase were due to i.p. formation of these factors.

The effluent samples showed a significant chemotactic activity. Blocking of PD fluid *ex vivo* indicated that the recruitment of neutrophils was mediated by either C5a or CINC-1. In order to quantify the C5a-dependent chemotactic activity, CINC-1 was blocked *ex vivo* before measuring chemotaxis. As expected, the remainder of chemotactic activity in animals treated *in vivo* with anti-C5 was virtually abolished at all time points. In the untreated group, *ex vivo* blockade of CINC-1 resulted in a progressive increase in C5a-dependent chemotaxis over time. This indicates that the neutrophil chemotaxis in the peritoneum is induced by complement activation and, probably, first C5a and, later, CINC-1 release from resident mast cells, macrophages, or mesothelial cells. The observed parallel release of CINC-1 and histamine may have been the result of mast cell degranulation. However, the histamine levels were low and it cannot be excluded that the increase over time could have been caused by diffusion from the vascular bed.

The present data suggest that there is a direct effect of the PD fluid on the peritoneal tissues or cells. Possible mechanisms include the dilution of the intraperitoneal fluid, changing the protein and cytokine levels, and the traumatic effects of distension and

osmosis on the peritoneal wall. The mechanisms behind the intraperitoneal activation of complement and coagulation are unknown. According to several clinical studies [72, 73], complement activation and coagulation [36] also take place continuously in CAPD patients. It has been speculated that the peritoneal inflammatory reaction in CAPD patients including complement and coagulation system activation, may be induced by endotoxins that diffuse from the gastrointestinal tract. Transmural migration of intestinal flora has been suggested as one of the patomechanisims underlying the peritonitis that develops in patients treated with PD [55]. One other possible mechanism behind the inflammatory response in the peritoneum could be the effect of a neurogenic inflammation. It is known that injury or chemical stimulation of the sensory nerve endings leads to the release of neuropeptides. The released neuropeptides then cause vasodilation and increased vascular permeability, probably by binding to the NK_1 receptors on the vascular endothelium, resulting in a condition known as "neurogenic inflammation" at and around the site of stimulation [74, 75]. Among the various neuropeptides, substance P is believed to be the major mediator of neurogenic inflammation. In addition to the direct effect of substance P on the microvasculature, degranulation of mast cells has also been demonstrated to contribute to the symptoms of neurogenic inflammation [76]. Morphological studies have provided visual evidence which confirms the close anatomical association between mast cells and nerve fibers, especially those containing substance P [77, 78]. Mast cell degranulation has also been documented in various *in vivo* models of neurogenic inflammation of the skin and gut [79]. We still don't know the mechanisms behind the activation of complement and coagulation cascades, but the complement activation seems to get engaged earlier in the course of events.

Intraperitoneal injections and indwelling catheters represent the two main PD techniques used in animals. Available data do not indicate that one model induces less inflammation than the other and there is no consensus on which model is more suitable [80]. According to the only published comparative study [80], dialysate cell counts, submesothelial fibrosis, and peritoneal transport properties were identical for injections and catheters after 1 month of daily exposure to hypertonic PD solution.

In the present study, the differences between the two models was evident from the beginning of the experiments, with the already elevated neutrophil and TAT levels in the catheter model. There was also a difference in D/D_0 glucose between the two models, indicating a higher rate of glucose diffusion from the peritoneal cavity of the

catheter model. However, at 2 hours, i.p. volumes did not differ significantly between the models. Unpublished data on the two models show that between 2 and 4 hours of dwell, i.p. volumes increase significantly in the injection model, while there is a tendency towards a decrease in the catheter model. Consequently, in the catheter model, the net reabsorption of fluid starts earlier. This is consistent with the lower glucose concentration found in the present study at 2 hours and indicates a larger available vascular surface area in the catheter model. Consequently, there is evidence that the two models differ also in terms of transport parameters. In the case of a single PD fluid exposure, the catheter model may be more relevant to clinical PD than the injection model, although the effects of the catheter per se in humans have not been clearly defined.

Effects of C5a blockade in peritoneal dialysis

In *paper II*, C5 blockade increased the ultrafiltration and reduced the early activation of the coagulation system. The tendency towards an improved retention of glucose during C5 blockade, though not significant, suggests that one mechanism behind the improved ultrafiltration is a reduction in glucose transport. This suggestion is supported by the finding of a significant positive correlation between glucose concentration and ultrafiltration, suggesting a connection between the increase in ultrafiltration caused by the C5 blockade and a reduction in the glucose transport from the peritoneal cavity. This may imply that C5a blockade inhibits inflammatory microvascular dilation and thereby reduces capillary flow velocities. C5a, in fact, has a vasodilatory effect on rat visceral arterioles mediated by nitric oxide [81] and nitric oxide blockade has been demonstrated to improve ultrafiltration [82]. Since glucose diffusion is much more flow-dependent than ultrafiltration, a reduction in capillary flow would decrease the out-diffusion of glucose [9], thus increasing the durability of the osmotic gradient and, secondarily, the cumulative ultrafiltration volume. An increase in glucose transport is a common mechanism behind ultrafiltration failure in PD patients, and blockade of C5a may therefore be a basis for increasing the efficiency of PD. However, there are many problems associated with the use of blocking antibodies raised against C5a in treating human patients. Immunogenicity is a common problem and proteins are expensive to manufacture, very susceptible to degradation by proteases in serum or the gastrointestinal tract, and generally display poor pharmacokinetic properties [31]. There are no specific low molecular weight complement-blocking drugs available.

Levels of the TAT-complex increased over time in both C5-blocked and unblocked animals, indicating that activation of the coagulation system was induced by the PD dwell. C5 blockade significantly reduced the initial levels of TAT. The demonstrated increase in ultrafiltration was paralleled by a significant reduction in levels of TAT in the samples taken immediately after initiation of the dwell. This indicates that C5a may be involved in mechanisms that initiate coagulation. Generation of the membrane attack complex during complement activation is known to induce pro-coagulant activity in platelets and endothelial cells, leading to coagulation [83], and C5 blockade inhibits formation of C5b, which is necessary for the assemblage of the membrane attack complex. At later time points during the dwell, there were no significant effects of C5a blockade, suggesting either that additional, C5a-independent, mechanisms of coagulation were at work or that the coagulation process was completed i.p. and was limited by the i.p. supply of coagulation factors.

The heparin effect in peritoneal dialysis

In *paper I*, we evaluated the effect of LMWH on PD, complement, and coagulation cascades and compared the two main techniques for i.p. fluid administration in animal studies, in the injection model, and in the catheter model.

Low-molecular-weight heparin showed the ability to block activation of the coagulation system and to reduce the i.p. chemotactic activity related to C5a, at the same time as increasing the ultrafiltration volumes. The two experimental models differed with regard to LMWH effects on neutrophil recruitment, the formation of C3a, and transport parameters. Complement activation and neutrophil recruitment were inhibited by LMWH only in the injection model, despite the fact that the reaction to PD fluid exposure was similar in both models. Addition of LMWH to the PD fluid, however improves ultrafiltration, inhibits formation of thrombin, and potentially blocks C5a activity in both models.

According to established theories the alternative pathway, most probably involved in the present experiments, is activated by surfaces lacking the complement inactivators present in normal cell membranes. The PD catheter is the obvious provider of such a surface. This would in part explain the complement activation in the catheter model. But, complement activation also occurred in the injection model, suggesting other, yet unknown, mechanisms.

Since C3a-desArg was not significantly affected in the catheter model, LMWH probably interacted directly with C5a, or else, C5a-desArg formed downstream of the C3 activation. This assumption was supported by the *in vitro* blocking effect of LMWH on C5a. Direct inhibition of C5a by different heparins has been observed in chemotaxis assays *in vitro* [60]. The magnitude of the LMWH-mediated increase in *paper I* was similar to that induced by blocking C5 in *paper II*. Consequently, it appears that C5a blockade may be the dominant mechanism behind the acute effect of LMWH on ultrafiltration. There are, however, several additional properties of heparin that may be involved, such as its ability to inhibit coagulation or its binding of angiogenic growth factors [59, 61].

Mechanisms behind the release of CINC-1 during peritoneal dialysis

The third study (*paper III*) was initiated to examine the possible role and involvement of mast cells in the acute peritoneal inflammatory response to PD fluids. The results demonstrate that complement activation plays a pivotal role in the early response to PD fluid exposure. The resulting intraperitoneal release of CINC-1 and the recruitment of neutrophils depend on the complement activation, since in our experiments, decomplementation with CVF virtually eliminated those responses.

Complement activation leads to the formation of several potent mediators of inflammation, C3a and C5a being the most important. In *paper II*, the CINC-1 release was not affected by C5a blockade. Therefore, it is most probably mediated by C3a, which is formed in larger quantities than C5a during complement activation. C3a is a potent anaphylatoxin with the ability to activate a number of cellular processes, sharing with C5a the ability to activate mast cells.

One hypothesis behind the present study was that the CINC-1 release induced by PD fluid is executed by mast cells, stimulated by C3a. Doxantrazole is, according to the literature, an efficient blocker of both mucosal and connective tissue mast cells [84, 85], although its specificity for mast cells has not been documented. There are several ways in which the mucosal mast cells differ from the connective tissue mast cells. Most importantly, their granulae contain different proportions of the various mediators. Histamine quantities are much lower and the proteoglycans differ structurally from those of connective tissue mast cells [86]. While histamine is a good marker of connective tissue mast cell secretion, RMCP-2 is specific to mucosal mast cells.

In our study, Doxantrazole reduced the neutrophil recruitment and showed a tendency towards blocking the CINC-1 release without significantly lowering the histamine or RMCP-2 levels. The generally low histamine and RMCP-2 levels following PD fluid infusion indicate that neither the connective tissue mast cells, nor the mucosal mast cells were activated by the PD fluid exposure. Unlike findings in allergic reactions, mast cells are rarely seen to degranulate during autoimmune or inflammatory processes. Instead, they appear to undergo ultrastructural alterations of their electron-dense granular core, indicative of secretion, but without overt degranulation, a process that has been termed "activation" or "intragranular activation". Such subtle activation may be associated with the ability of mast cells to selectively release some mediators [87]. However, this finding is inconclusive and needs to be studied further. The slight increase in histamine concentration seen during the dwell in our study was probably due to leakage from the circulation since histamine is a relatively small molecule that clears rapidly over the peritoneal membrane. Plasma concentrations were considerably higher than the i.p. concentrations, making up a positive concentration gradient for this clearance. Similar conditions were in place for RMCP-2 clearance. The normal concentrations of RMCP-2 in rat plasma are in the range of 50 ng/ml [88], which is 25–50 times higher than those measured i.p. in *paper III*.

Consequently, there was no robust evidence for mast cell activation. This implies that, instead, Dox blocked CINC-1 release from cells other than mast cells. However, mast cell blockers are generally not well characterized with regard to mechanisms of action and the documentation on Dox is no exception to this rule. The other main candidates among peritoneal cells for CINC-1 release are macrophages [89] and mesothelial cells [29], although little information is available in the literature on this subject. These cells are capable of inducing neutrophil migration and contributing to the high CINC-1 levels in our model; however, the mechanisms connecting them to the PD fluid response are unknown. Data from intraperitoneal wash-outs in the beginning of dwells neither showed significant effects on CINC-1 levels, nor on neutrophil numbers after 4 hours dwell although the wash-out reduced the macrophage population by at least 75%. This turns our attention towards the resident cells. The lacking evidence for mast cell degranulation implies that the importance of mesothelial cells should be evaluated.

The effect of glucose degradation products on CINC-1 release and neutrophil function

The results of the last study (*paper IV*) indicate that GDPs formed during heat sterilization of PD fluids have inhibitory effects on important inflammatory mechanisms of experimental peritonitis in the rat. The recruitment of neutrophils to the peritoneal cavity after inducing experimental peritonitis with opsonized zymosan was significantly diminished by heat-sterilized PD fluid compared with filter-sterilized PD fluid. This effect can possibly be explained by a parallel inhibition of the release of the chemokine CINC-1. When inducing experimental peritonitis with unopsonized zymosan, on the other hand, the GDPs influenced neither the recruitment of neutrophils, nor the levels of CINC-1 present i.p. Both types of zymosan induced a similar degree of i.p. complement activation, measured as an increase in the concentration of C3a-desArg, and the level of this complement activation was not influenced by the GDP concentration of the PD fluid.

The reason for the increased sensitivity of CINC-1 release to GDPs remains obscure, but the finding is in line with results of a number of earlier studies on GDP toxicity. Exposure of human mesothelial cells to heat-sterilized PD fluid significantly impaired cell viability and the capacity for generating monocyte chemoattractant protein-1 (MCP-1) and production of IL-6 [90, 91] compared with filter-sterilized PD fluid [92]. Glucose degradation products hypothetically exert a wide range of inhibitory effects on cells by carbonyl-induced cross-linking of molecules such as surface proteins, intracellular proteins, and nucleic acids. For example, formaldehyde, a GDP species and a well-known fixative, easily penetrates the cell membrane and immediately cross-links several intracellular components. The complement system, though based on a series of catalytically active proteins, was not significantly affected by GDPs in *paper IV*.

It is obvious that the two types of zymosan particles differ concerning their interaction with the immune system. The fact that opsonized zymosan reduced the ultrafiltration capacity of the PD fluid compared with unopsonized zymosan, indicates a difference also in the interaction with microvascular perfusion and/or permeability.

Consequently, the results of the last study (*paper IV*) indicate that GDPs affect mechanisms of neutrophil recruitment that are independent of i.p. complement

activation, but probably involve chemokine release. The neutrophil respiratory burst response is significantly inhibited by GDPs if the neutrophils are present i.p. when the PD fluid is injected, demonstrating the importance of the rapid detoxification of GDP-containing PD fluid in vivo. Our results also suggest that some parts of the immune response, including the complement system, are not affected by GDPs. It also appears as if some routes of chemokine activation, such as that utilized by unopsonized zymosan, may be fairly insensitive to the GDP effects although other pathways, such as the one utilized by opsonized zymosan, may be significantly inhibited by GDPs. Hypothetically, GDP effects on the immune response may favor certain pathogens, including those that do not activate complement. There is a range of microorganisms that inhibit complement activation and / or opsonization but neither of those is among the common pathogens of PD-associated peritonitis.

CONCLUSIONS

The main aim of the present thesis was to elucidate the regulatory mechanisms behind the acute inflammatory response associated with PD fluid instillation in the presence or absence of experimental peritonitis. Clinical studies have demonstrated that i.p. complement and coagulation systems are activated in PD patients and that PD maintains a state of i.p. inflammation. Some PD fluids also have anti-inflammatory properties, possibly interfering with the peritoneal immune response. Data from this thesis show that _

- complement activation is a very early step in the acute reaction to PD. It reduces ultrafiltration through the action of C5a and it probably mediates the downstream events that lead to recruitment of inflammatory cells to the peritoneal cavity. The cells involved in the release of CINC-1 later in this sequence are possibly mesothelial cells. However, the importance of the resident macrophages cannot completely be ruled out and remains to be investigated further.

- blockade of C5 leads to an increase in ultrafiltration, probably by a mechanism that involves a reduction in glucose transport. This effect may form the basis for increasing PD efficiency.

- supplementation of LMWH to the PD fluid improves ultrafiltration, inhibits formation of thrombin, and blocks C5a activity.

- the neutrophil respiratory burst response is inhibited by GDPs present in heat sterilized PD fluids if the neutrophils are present intraperitoneally when the PD fluid is injected.

- GDPs in PD fluids also inhibit the recruitment of neutrophils to the peritoneal cavity by a mechanism that is not dependent on complement activation, but that probably depends on cytokine CINC-1 release.

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