Regulation of Cytokine Release from Peritoneal Macrophages of Patients on Continuous Ambulatory Peritoneal Dialysis

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Regulatie van de afgifte van cytokinen door peritoneale macrofagen van patiënten met Continue Ambulante Peritoneale Dialyse

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List of Abbreviations

ACTH	Adrenocorticotropic hormone
ADCC	Antibody dependent cellular toxicity
ATP	Adenosine triphosphate
C_3	Complement factor 3
cAMP	Cyclic 3', 5' adenosine monophosphate
CAPD	Continuous Ambulatory Peritoneal Dialysis
CNS	Central nervous system
CSF	Colony stimulating factor
CRF	Corticotropin releasing factor
DNA	Deoxyribonucleic acid
E. coli	Escherichia coli
ELISA	Enzyme linked immunosorbent assay
GM-CSF	Granulocyte-macrophage colony stimulating factor
5-HETE	5-hydroxy eicosatetraenoic acid
5-HPETE	5-hydroperoxy eicosatetraenoic acid
ICAM-1	Intracellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRMA	Immuno radiometric assay
LAF	Lymphocyte activating factor
LPS	Lipopolysaccharide
LT	Leukotriene
MHC	Major histocompatibility complex
mRNA	messenger Ribonucleic acid
NAP-1	Neutrophil attractant/activation protein-1
P 450	Cytochrome P 450
PAI	Plasminogen activator inhibitor
PG	Prostaglandin
PMN	Polymorphonuclear cell
RIA	Radio immuno assay
TXA_2	Thromboxane A_2
TNF	Tumor necrosis factor

Chapter 1

General Introduction

1.1. General introduction

Continuous Ambulatory Peritoneal Dialysis (CAPD) is a modality for treating patients with end stage renal failure (12). Toxic metabolites retained in the body due to renal failure, are removed by infusing dialysis fluid into the peritoneal cavity. During a dwell time of 4-8 hours of the dialysis fluid, substantial amounts of retained toxic metabolites diffuse into the peritoneal cavity. After this, the dialysis fluid is drained. Besides uraemic metabolites, the effluent dialysate has been found to contain leucocytes, mainly macrophages. When CAPD is complicated by an episode of peritonitis, usually caused by contamination of the peritoneal cavity with microorganisms during the exchange of dialysis fluid, the cellular composition of the drained dialysate is drastically changed. There is a large increase in the number of leucocytes and the population is now predominantly composed of polymorphonuclear cells (PMN's). The macrophages, which are present, are probably in some stage of activation. The infiltration of tissues by PMN's is commonly found during acute inflammatory processes. Inflammatory reactions are thought to make a major contribution to the elimination of microbial invaders, or in general to the defence against injury. The fact, that in CAPD related peritonitis, the leucocytes are predominantly PMN's, does not imply, that macrophages are of minor importance in this condition. Though outnumbered by PMN's, the number of macrophages is also increased considerably during peritonitis. (see chapter 4). In experimental infection with E. coli, in mice, monocytes were found to migrate into the inflammatory site simultaneously with PMN's after induction of the infection (10). In fact, symptoms like fever, that can accompany acute infection-induced inflammatory reactions, dominated by PMN's, are predominantly due to macrophage derived mediators (see later). Macrophages originate from circulating blood

monocytes migrating into the tissues. Both monocytes and macrophages are part of the mononuclear phagocyte system (8,9). In spite of the morphological, biochemical and functional heterogeneity of macrophages and monocytes, no substantial evidence has been provided for the existence of distinct mononuclear phagocyte lineages (6). The presence of specific signals in the environment (inflammatory stimuli for instance) determines to which functional state the macrophages develop (1). This provides the rationale for the comparison of macrophages collected during an episode of peritonitis with those harvested during an infection-free period.

During the past decade, our understanding of the pathophysiology of inflammation has been deepened, with the discovery of the cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and Tumor Necrosis Factor (TNF) (5, see also chapter 3). These proteins are secreted at the inflammatory site, mainly by mononuclear phagocytes and have a wide array of proinflammatory effects. In fact, administration of these cytokines is able to induce inflammatory reactions. Cytokines are not only mediators of local inflammation, but they can also gain access to the circulation, thereby exerting their actions at distant organs, as such resembling endocrine hormones. In this way, cytokines orchestrate the body's response to infection (or any other inflammation inducing injury). For instance, fever is induced by their action on the preoptic hypothalamic area in the brain, and they stimulate the synthesis of acute phase proteins by the liver.

Since inflammatory reactions can also have damaging effects a high response, in terms of secretion of pharmacologically active compounds, may result in serious injury. This applies to the release of arachidonic acid metabolites (prostaglandins, leukotrienes) and cytokines. Prostaglandin E_2 (PGE_2) and Prostacyclin (PGI_2) have been shown to have inhibitory effects on several macrophage functions (3). These substances are secreted by various cells present at the inflammatory site, including macrophages. The same stimuli (e.g. endotoxin) promote both cytokine and prostaglandin synthesis from macrophages, suggesting that prostaglandins may play a role in the regulation of macrophage functions in vivo (11). Prostaglandins are derivatives of arachidonic acid, which is metabolized either by the enzyme cyclooxygenase to give rise to prostaglandins and thromboxanes or by the enzyme 5-lipoxygenase to give rise to various leukotrienes. (Metabolites of arachidonic acid are currently named as eicosanoids). (3) It is well known that prostaglandins can enhance the vascular component of inflammation by contributing to increased blood flow and vascular permeability, which are typical of inflammatory reactions (11). These findings

show that prostaglandins play a dual role in inflammation in that they promote the vascular component of inflammation (proinflammatory effects), whereas the macrophage component of inflammation is inhibited (antiinflammatory effects). Increased blood flow and vascular permeability lead to a rise in the transport of larger serum molecules, such as complement and immunoglobulins out of the vessels. Evidence suggests that leukotrienes, in contrast to prostaglandins, exert predominantly stimulatory actions on macrophages (3). They are potent chemotactic compounds, contributing to migration of leucocytes to inflamed tissues. In contrast to the cytokines IL-1, IL-6 and TNFα, eicosanoids only act as local hormones (autacoids) (see chapter 3).
This thesis deals with the relationship between prostaglandins and cytokines released by peritoneal macrophages from CAPD patients.It will be shown that the changes which macrophages undergo during episodes of peritonitis are reflections of the functional regulatory connections which exist between cytokines on the one hand and prostaglandins on the other.
The cytokines that will be discussed, are IL-1 and TNFα.

1.2. Aim of the thesis

The foundation of this thesis was laid in 1982 when we made the first studies of the production of inflammatory mediators by macrophages collected from effluent dialysate of patients on Continuous Ambulatory Dialysis (CAPD).
During the first experiments, these cells, harvested from infection-free patients, proved to be suitable for immunopharmacological studies (4). When macrophages were collected when CAPD was complicated by an infectious peritonitis, the cells exhibited some striking differences in comparison with macrophages that were collected when the patients were infection-free. Notably the finding that the release of prostaglandins and the intracellular cAMP levels were decreased in macrophages collected during an episode of peritonitis, was intriguing (2). While prostaglandins are well known for their proinflammatory properties they can have antiinflammatory properties as well thought to be mediated by stimulating intracellular cAMP levels (3). At that time emphasis was put on the proinflammatory properties of prostaglandins, while the relevance of the antiinflammatory properties to in vivo conditions was sometimes questioned. The finding that macrophages from an inflammatory environment

showed a decrease in the release of prostaglandins, might indicate, that prostaglandins fulfil antiinflammatory-immunomodulatory rather than proinflammatory functions with regard to these macrophages during the acute phase of an inflammation. A decrease in the release of the anti-inflammatory prostaglandins could allow macrophages to become "activated" during an infection (7). To test this postulate I proposed, in 1983, to investigate the secretion of the proinflammatory "factor" later identified as interleukin-1: IL-1, by using peritoneal macrophages isolated from CAPD patients during peritonitis and during an infection-free period. The following questions had to be answered: 1) Is the decreased secretion of the antiinflammatory prostaglandins, as found during peritonitis, associated with increased secretion of the proinflammatory IL-1? 2) Do prostaglandins have inhibitory effects on IL-1 secretion? These questions underlie the studies of this thesis.

IL-1 secretion by peritoneal macrophages from infected patients as compared to infection-free patients, is described in *chapter 5*.

In chapter 6, the relationship between the secretion of IL-1ß and that of prostaglandins (PGE_2, PGI_2) was studied.

In chapter 7, the in vitro secretion of Tumor Necrosis Factor α (TNFα), an other cytokine with proinflammatory properties, by peritoneal macrophages from infected patients is compared with that of infection-free patients.

To what extent the release of IL-1ß and TNFα from peritoneal macrophages is affected by exogenous PGE_2 or the cyclooxygenase inhibitor indomethacin, is discussed in *chapter 8*.

These findings are summarized *in chapter 9*. Furthermore the relevance of these findings with regard to the acquisition of functional competence by peritoneal macrophages during infection, is discussed.

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Chapter 2

Continuous Ambulatory Peritoneal Dialysis (CAPD)

2.1. Introduction

In 1976 Popvich et al. introduced Continuous Ambulatory Peritoneal Dialysis (CAPD) as a new modality of peritoneal dialysis for treating patients with end stage renal failure (6). Until then, a different modality of peritoneal dialysis, Chronic Intermittent Peritoneal Dialysis, developed by Fred Boen working in Amsterdam and Seattle in the early sixties (2), occupied a modest place in the treatment of end stage renal failure; the majority of patients were treated with Chronic Intermittent Haemodialysis or kidney transplantation. Chronic Intermittent Haemodialysis was developed in the sixties into a routine treatment for patients with chronic renal failure as a result of the pioneering work of Willem Kolff in the Netherlands during World War II. (1) For the majority of patients chronic intermittent haemodialysis was preferred in the sixties and seventies, mainly because of its greater efficiency as compared with Chronic Intermittent Peritoneal Dialysis. An upturn in peritoneal dialysis occurred after the introduction of CAPD in 1976. In CAPD after infusion of dialysis fluid, usually 2 litres, retained metabolites diffuse from the blood to the peritoneal cavity during a dwell time of 4-8 hours, after which the dialysate is drained. After drainage, fresh dialysis fluid is infused. In this way the patient changes 4 times a day dialysis fluid, giving an efficiency comparable to chronic haemodialysis. Initially, CAPD was frequently complicated by peritonitis due to contamination of the peritoneal cavity with microorganisms. (3) Since 1978, when dialysis fluids began to be delivered in "plastic" (PVC) bags, an increasing number of patients have been treated with CAPD (5). After infusion of fresh dialysis fluid, the empty bag remains connected to the catheter and is not disconnected until

the dialysate drainage after 4-8 hours dwell time is completed. This procedure allowed reduction in the number of disconnections and was aimed to reduce the risk of infection. Nevertheless, peritonitis rates remained high and many efforts were made to develop new devices in order to reduce the risk of infection (4). CAPD related peritonitis accounted for frequent hospitalizations and high drop out rate from CAPD. In spite of the morbidity of peritonitis, a growing percentage of the dialysis population received CAPD. In comparison with haemodialysis, CAPD induces no disequilibrium syndrome, allows more liberal fluid intake and it enables the patient to perform home dialysis without the requirement for a partner and adjustments in the home. CAPD may be preferred for patients with cardiovascular disease and diabetes (4). Certainly, reducing the rates of peritonitis could further increase the use of CAPD.

2.2. Experience with CAPD related peritonitis in University Hospital "Dijkzigt"

Two hundred fifteen episodes of peritonitis developed in 137 patients who started CAPD in our centre from 1980-1991 (april). According to life table analysis, 66 percent of CAPD patients are estimated to develop the first episode of peritonitis within the first year of therapy (fig. 1). For the whole period, a total of 1.2 episodes of peritonitis per patient-year of observation occurred. These data reasonably agree with those reported in the National CAPD Registry of the U.S. (4). Remarkably, the peritonitis rate remains fairly constant until 1989, apart from fluctuations probably attributable to the low number of patients on CAPD in the early eighties, as shown in fig. 3. A constant peritonitis rate throughout this period was also reported by others, in spite of the fact, that many claimed a reduced peritonitis rate due to improved connection systems (4). As shown in fig. 3, peritonitis rate shows a sharp drop since 1989, when an growing number of CAPD patients in our centre started to use the TWIN BAG^{R} system (fig. 2). If this device, or another type of Y-set, is used, the fresh dialysis bag is connected just before drainage. This allows the draining fluid to flush the tubing which connects the dialysis bag and the catheter, before infusion of fresh dialysate. Many centres have reported a striking reduction in peritonitis rate, if an Y-set was used (7).

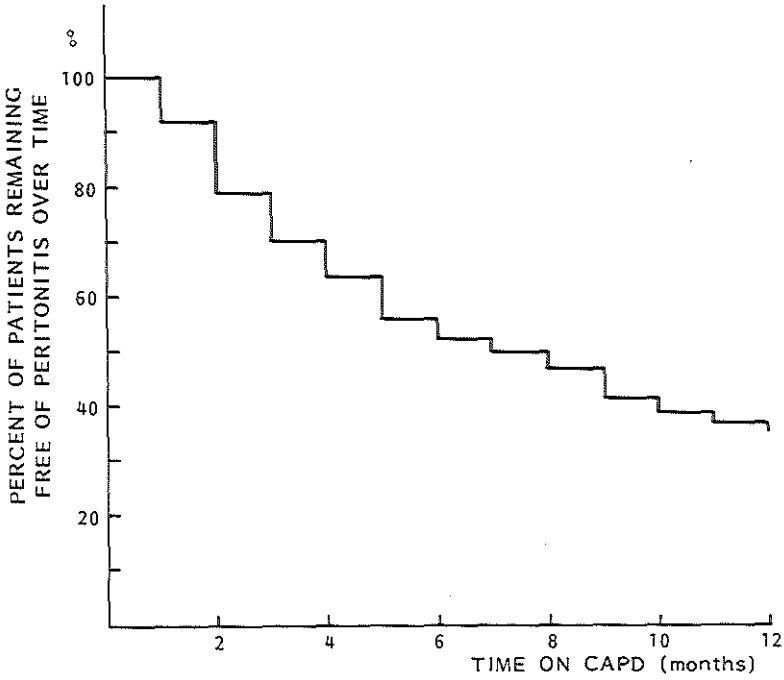

Fig. 1 Life table for time (months) to first episode of peritonitis (n=137).

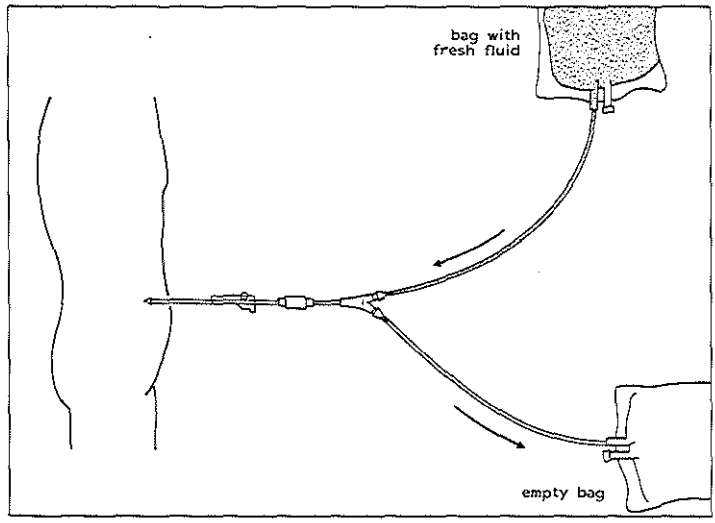

Fig. 2 Schematic drawing of TWIN BAG^{R} system.
After connection of the system to the peritoneal catheter, effluent dialysate drains into the empty bag, which allows the draining fluid to flush the connecting tubing before inflow of fresh dialysate. After the inflow has been completed, the system is disconnected.

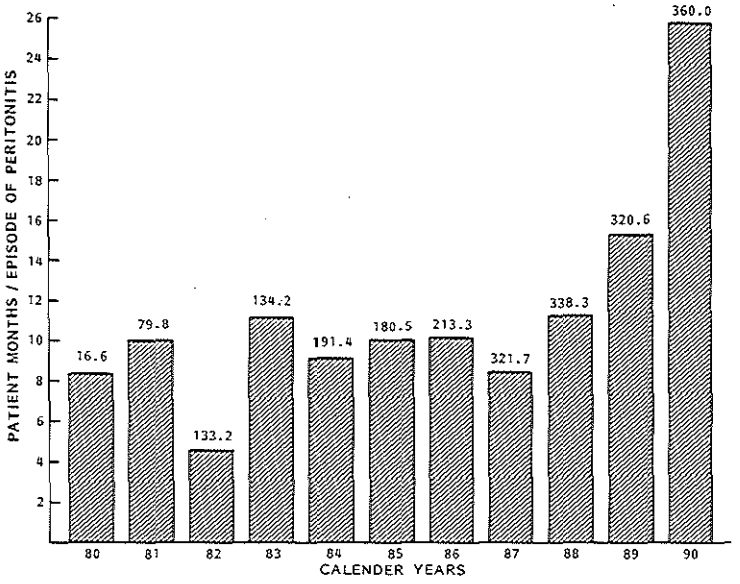

Fig. 3 Peritonitis incidence expressed as elapsed number of patient months per episode of peritonitis. The numbers over the bars indicate cumulative time on CAPD (patient months) per calendar year.

As shown in table 1, gram positive cocci are the most common causative microorganisms. These bacteria, probably coming from skin flora, enter the peritoneal cavity intraluminally or extraluminally via the catheter tunnel (8). Peritonitis caused by E. coli or multiple enteric organisms was usually related to intestinal perforation, as a result of diverticulitis or ischaemia.
It is worth mentioning, that none of the peritonitis episodes was associated with positive blood cultures.
As shown in table 2, the cause of peritonitis was established in 25 percent of peritonitis episodes. Peritonitis was classified as being caused by poor technique, when obvious faults were made.
Only peritonitis caused by bowel perforation was directly related to death.
12 percent of the patients dropped out from CAPD because of peritonitis. (table 3) Especially during the first years after the CAPD program was started in our centre, a relatively large number of patients dropped out from CAPD because they found frequency and morbidity of peritonitis (hospitalization, removal of peritoneal catheter) unacceptable. After 1985, peritonitis infrequently lead to drop out.

Table 1. Causative microorganisms of 215 peritonitis episodes.

microorganism	%
Staphylococcus epidermidis	23.3
Staphylococcus aureus	13.0
Streptococcus viridans	8.8
Other gram positive cocci	2.8
Diphteroids	4.2
Bacillus anitratum	8.4
Pseudomonas	7.4
Other gram negative rods (E.coli, Klebsiella)	3.3
Yeasts	3.3
Fungus	1.0
miscellaneous	0.5
no culture performed	0.5
multiple microorganisms	4.2
no growth	20.0

Table 2. Causes of 215 episode of peritonitis.

cause	%
exit-site infection or tunnelinfection	13.9
intestinal perforation	2.7
accidental system disconnection or system break	3.2
poor technique	4.1
unknown	75.3

As shown in table 3, the majority of peritonitis episodes were associated with mild symptoms and signs, like mild abdominal pain and turbid dialysate due to an increased number of leucocytes (predominantly neutrophil granulocytes). Peritonitis is almost invariably revealed by opalescence of the dialysate, which is always noticed, when the leucocyte count is greater than 100/mm^3 (8). In 13.5 percent of peritonitis episodes patients were gravely ill with high fever and severe abdominal pain. (table 3).

Table 3. Symptoms and complications in 215 episodes of peritonitis.

	%
mild symptoms	57.6
moderate symptoms	28.8
severe symptoms	13.5
removal of peritoneal catheter	28.6
drop out from CAPD	12

In 28.6 percent of peritonitis episodes the peritoneal catheter was removed (table 3). Especially in the first years after the start of the CAPD program, recurrence of peritonitis with the same microorganism (referred to as relapse), lead to removal of the catheter. The last few years, removal of the peritoneal catheter because of relapsing peritonitis was only occasionally required, due to the use of other antibiotics like vancomycin.
In conclusion, during the past decade treatment of peritonitis has been improved. In addition, during the last few years a substantially lower frequency of peritonitis could be achieved thanks to the development of a new connection system.

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Chapter 3

Macrophages and Mediators

3.1. The evolution of macrophages and cytokines

All living organisms must protect themselves against foreign invaders. In the animal kingdom phagocytosis is the oldest known mechanism by which unwanted material is removed. Phagocytosis is carried out by specialized cells, phagocytes, which are able to recognize and remove unwanted material. These cells have the ability to distinguish between "self" components and foreign materials, such as microorganisms (53). Equally important is their capability to discriminate between "normal" and "abnormal" self, which includes damaged, aged and neoplastic cells. (99). The ubiquity and importance of phagocytosis was recognized as early as 1883 by Elie Metchnikoff (99). Even unicellular eukaryotic organisms (protozoans) are capable of ingesting selectively particles from the environment for their nourishment (53). In primitive multicellular organisms, such as sponges, which lack a blood system, wandering amoeboid, phagocytic cells (referred to as archeocytes) digest and distribute the trapped food. These cells are also involved in self defence of the sponges (99). In metazoans such as jelly fish, sea anemones and corals, a distinctive, more orthodox defensive phagocyte is found. This amoebocyte participates in healing, tissue organisation and phagocytosis of foreign tissue. There are two types of phagocytic cells: one is involved in the response to foreign invasion and the other in feeding. Infiltration of amoebocytes occurs, which may be viewed as an inflammatory reaction (99). When, in the course of evolution, there was an increase in body size and complexity, a circulatory system with blood (haemolymph) evolved for the transport of food, metabolites and gases, thus allowing the phagocytes to be freed from nutritional duties. It is thought, that phagocytic cells migrated from the surrounding connective tissue into the haemolymph to become specialized defence cells (haemocytes). Mammalian phagocytes

(mononuclear phagocytes, granulocytes) are thought to have evolved from these haemocytes. The defence system of vertebrates was equipped with the sophisticated immune system for the highly specific recognition and memory of antigenic structures. This branch of the immune system relies on the highly specific antigen recognition by B- and T-lymphocytes. B-lymphocytes are the manufacturers of humoral antibodies. Although among invertebrates, in view of the presence of allogenic and xenogenic graft rejection, specific recognition occurs, there is no clear evidence, that invertebrates possess lymphocytes homologous to those of vertebrates (104). Rather, haemocytes and amoebocytes, which correspond to vertebrate macrophages, are involved. In vertebrates the non-specific and the more advanced specific branch of the defence system work in close cooperation. For instance, macrophages present antigen to T-helper lymphocytes in order to initiate immune response. Conversely, antibodies against microorganisms facilitate the recognition and final destruction of microbes by macrophages.

In recent years, the discovery of cytokines has shed light on the complex organisation of the host defence system of mammals. Among them, especially interleukin-1 (IL-1) and Tumor Necrosis Factor (TNF) have shown to play a key role in the communication between a wide variety of cells and tissues involved in defence (25). The mononuclear phagocyte (monocyte, macrophage) is the main source of these protein messengers. Due to their manifold activities, cytokines are able to integrate the various parts of the defence system to each other. IL-1 displays striking structural and functional similarities among the vertebrate classes. The same holds for TNFα indicating that these molecules have been highly conserved through vertebrate evolution. In recent years several attempts have been undertaken to study the phylogeny of cytokines from traces left by evolution in extant invertebrates. Several studies provide evidence, that cytokines are ancient signal molecules, whose evolutionary origin antedated that of the vertebrates (7). Thus, IL-1 activity was found in several species of tunicates (sea squirts), which are assumed to be evolutionary most closely related to the vertebrates. It is remarkable that IL-1 activity in these species was demonstrated using a murine thymocyte proliferation assay (LAF-activity). The IL-1 activity was neutralized by antisera directed against human IL-1 (8). TNFα activity could not be demonstrated in this species. Also in the starfish, belonging to the echinodermata, which are evolutionary also close to the vertebrates, IL-1 activity was found (9). Moreover, two forms could be distinguished with

biochemical characteristics similar to human and murine IL-1α or IL-1ß, suggesting, that the divergence of the α gene from the ß gene may have occurred more than 600 millions years ago (10). Haemocytes of mollusces proved to respond to human recombinant IL-1 and TNFα in a manner similar to that of human phagocytes (51). The effects of IL-1 were brought about, in part, via a stimulatory effect on the generation of TNF, suggesting the existence of a cytokine network (51). Moreover, immunoreactive IL-1 and TNF were demonstrated in the haemolymph. These findings suggest, that IL-1 and TNF have originated early in the course of evolution and that their function and structure have been retained. Recent findings indicate that even unicellular protozoans may possess cytokine-like molecules. In these organisms a structural and functional relationship between IL-2 and pheromones (mating inducing substances) has been found (87). These substances are in fact involved in the process of cell recognition. Also human IL-1ß was found to be an effective competitor of pheromone receptor binding.

During the last decade, vertebrates and invertebrates were found to share a number of molecules, which have a function in recognition and defence. For instance substances related to C reactive protein, which can carry out functions such as fixation of complement and binding to phagocytic cells, have been found in tunicata and the horseshoe crab (72). Neural cell adhesion molecules, which are, like the IL-1 receptor included in the immunoglobulin superfamily, have also been found in species of insects (72). Neural cell adhesion molecules participate in cell-cell interactions during neural morphogenesis. In general, cell adhesion proteins are involved in the interaction of many cell types, including those of the immune system. Furthermore, in vertebrates and invertebrates an increasing number of substances has been found during the last decade that serve as intercellular messengers in both the neuroendocrine system and the immune system. Opioid neuropeptides for example are involved in the regulation of immune-inflammatory reactions (97). Neuropeptides induce the release of the cytokines IL-1, TNFα and IL-6. (69) Conversely, these cytokines induce febrile responses, somnolence and the secretion of corticotropin releasing factor from hypothalamic neurons (11). On the ground that substances resembling hormonal peptides, neuropeptides and receptors of vertebrates have been identified in protozoans and even in bacteria and higher plants, the paleocentric or unification theory was proposed (92). For instance, materials resembling adrenocorticotropic

hormone (ACTH), ß-endorphin and insulin are present in microbes. In higher plants, substances related to interferon, insulin and thyrotropin releasing hormone (TRH), among others are found. The paleocentric concept implies, that the molecules of intercellular communication arose much earlier in evolution than the endocrine, nervous and immune systems of multicellular organisms. This intriguing theory is also consistent with the finding that in man (and other mammals) several hormones and receptors are produced by cells of diverse organ systems. It raises the question why certain signal molecules and their association with specific functions have been conserved throughout evolution. Conceivably, this may be attributed to the fact that every aspect of a signal system including synthesis, degradation and receptor mediated events, is stereoselective. Changes in structure or confirmation of the bioactive portion in one component of the signal system need to be accompanied by compatible changes in each of the other components, in order for the system to be operational as a whole. The fact that the simultaneous occurrence of changes on the corresponding genes of the components of a signal system is unlikely, may account for the tendency of ancient communication systems to remain conserved (97).

In conclusion, there is ample evidence, that human macrophages have retained ancient defence mechanisms including phagocytosis, encapsulation and cell recognition. Similarly, molecules related to the cytokines IL-1 and TNF may have originated early in the course of evolution. Especially due to their capability to produce cytokines, macrophages play a key role in integrating the more advanced specific immune system, that evolved in vertebrates, into the primordial non-specific host defence mechanisms. Moreover, cytokines constitute the connecting link between the immune system and other organ systems.

3.2. Macrophages

3.2.1. Introduction

Macrophages are involved in many aspects of the specific and non-specific branch of the host defence system. In addition to their well known capability to destroy microbial invaders and tumor cells, these cells can have many other functions such as regulation of (acute) inflammatory

reactions, recognition of foreign cells to initiate specific immune response (antigen processing), scavenging of cellular and molecular debris and regulation of coagulation and fibrinolysis (2,76). Both the capability of macrophages to handle extracellular proteins and to secrete a host of substances, enables these cells to carry out such a variety of functions.

3.2.2. Origin of macrophages

Tissue macrophages are derived from circulating blood monocytes, which in turn arise from their bone marrow precursors promonocytes and monoblasts. These cells together make up the mononuclear phagocyte system (34,35). After monocytes have entered the tissues to become macrophages, they have the potential to acquire a variety of different functional attributes depending on signals the cells receive from the environment. In the absence of inflammatory stimuli, the immigrated macrophages develop to resting tissue macrophages (2). Thus, the mononuclear phagocyte is a highly versatile, multipotential cell, whose differentiation in the tissue is governed by the presence of regulatory signals in the environment. The developmental stages of mononuclear phagocytes can be distinguished by their peroxidase activity patterns. The presence of peroxidase-positive granules in exudate macrophages, which are monocytes recently migrated from the circulation to the site of inflammation, can be employed to distinguish these cells from resident macrophages (36). With electron microscopy the localisation of peroxidase activity can be detected. Resident and exudate-resident macrophages have peroxidase activity in the nuclear envelope and the rough endoplasmic reticulum, in contrast to monocytes and exudate macrophages, which have only peroxidase activity in the lysosomal granules. In monoblasts and promonocytes, peroxidase activity can be detected in endoplasmic reticulum, nuclear envelope, Golgi apparatus and lysosomal granules.

3.2.3. Macrophage function and activation

Since most functions of tissue macrophages are not constitutively expressed, further development in tissues is required (2). Originally, the term macrophage activation was introduced for the acquisition of enhanced anti-microbial activity (22,56,80). Later, it was shown that macrophages obtained from animals infected with intracellular bacterial parasites, had acquired also enhanced antitumor activity, which lead to the unfounded

assumption that the induction of one functional manifestation automatically implies the induction of others. In fact, the acquisition of one physiologic property may be accompanied by downregulation of other properties. Therefore, macrophage activation represents the acquisition of defined biochemical and physiological properties, which, by their combined action, enable the macrophages to complete a given function (2). There is convincing evidence that activation occurs in a number of definite steps (41). The concept that macrophages are activated in (two) steps, was put forward by Gordon et al. (41). These authors showed that macrophages harvested from mice after an intra-peritoneal injection of LPS, released little plasminogen activator. Subsequent phagocytosis of latex or bacteria triggered these "primed" macrophages to release high levels of plasminogenactivator, as long as the phagocytic stimuli persisted. This concept has been proven to be very fruitful. It applies to the development of many functional responses (2). For instance, in order to develop full tumoricidal potential, macrophages need first to be primed by the lymphocyte secretory product interferon γ. Subsequent stimulation with LPS "triggers" actual secretion of lytic substances. (fig. 1).
A model of two-step activation of macrophages for tumoricidal activity is shown in fig. 1.

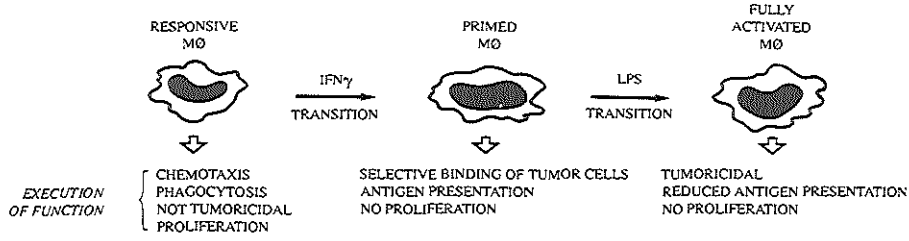

Fig. 1 Responsive (young) macrophages acquire the capacity to capture tumor cells after exposure to interferon-γ. When these primed macrophages are subsequently stimulated with LPS they undergo transition to the next stage, thereby acquiring the capacity for the secretion of lytic mediators such as Tumor Necrosis Factor and cytolytic protease (from reference 2).

3.2.4. Macrophages and resistance to infection

Macrophages play a key role in defence mechanisms against microorganisms. Following recognition by the macrophages, microorganisms undergo adsorption and/or internalization and are finally destroyed by the intracellular release of lytic substances including reactive oxygen intermediate species (e.g. H_2O_2) and nitric oxide (44). Principally, macrophages recognize microorganisms via the action of opsonins, such as immunoglobulins, complement C_{3b} and fibronectin, which bind to specific sites of both the macrophage and the microorganism. In some cases, microbial surface structures are directly recognized without the intervention of these humoral factors (2). The cellular immune system is involved in acquired resistance to infections with facultative intracellular micro-organisms, including Brucella, Salmonella and Listeria (22,80). The capability of macrophages to destroy these microorganisms is greatly enhanced by the secretion of soluble factors, such as interferon-γ by T-lymphocytes, which are specifically sensitized to a given microorganism. Once activated in this fashion, the macrophages were found in a number of cases to have also an increased capacity to destroy unrelated micro-organisms.
Finally, macrophages may enhance resistance to infections by amplification and orchestration of inflammatory reactions. This is discussed in the section on cytokines.

3.2.5. Peritoneal macrophages of CAPD-patients

Between 1 million and 40 million leucocytes can be harvested from effluent dialysate of "morning bags" from CAPD patients (40,70). In the uninfected patients, the leucocyte population is composed of roughly 85% macrophages, 5% lymphocytes and 5% Polymorphonuclear cells (PMN's). CAPD peritoneal macrophages appear to be relatively immature mono-nuclear phagocytes, freshly derived from bone marrow, as judged by their response to chemotactic stimuli and eicosanoid precursor uptake (40). Usually, peritoneal macrophages from CAPD patients are compared with such cells from healthy women undergoing laparoscopy, or with peripheral blood monocytes, either from uraemic or non uraemic subjects. In this connection, the following points are of relevance. First, because of the continual removal of the leucocyte populations by the exchange of dialysis fluids, the number of macrophages (and the concentration of opsonins), is

so low, especially after the inflow of fresh dialysate, that microorganisms may escape macrophages (16). Second, the high dextrose contents and the low P_H of the dialysis fluid, which persists for 30-60 minutes after dialysate inflow, might impair macrophages. Indeed, culturing macrophages in vitro in the presence of standard dialysis fluids, proved to impair viability and antibacterial capacities of these cells (18,29). However, peritoneal macrophages, after isolation incubated in dialysate free culture media, displayed normal phagocytic and bactericidal capacities (101). Third, macrophages isolated from patients on standard CAPD are found to be relatively immature cells. They could be characterized as exudate macrophages, resident cells being very scant (17). We found, that peritoneal macrophages from CAPD-patients showed resemblance to starch elicited rather than to resident peritoneal macrophages from rats (15). It is suggested, that such immature cells could have a diminished antimicrobial activity (16). However, this is less clear and in fact, immature macrophages might be functionally more effective in local defence (66). Fourth, peritoneal macrophage function may be affected by uraemia (33).
Table 1 summarizes several functions of peritoneal macrophages from CAPD patients (66).
In conclusion, there is ample evidence, that peritoneal macrophages of CAPD patients are well functioning leucocytes in many respects. It cannot be ruled out, however, that low density of macrophages and the unphysiological composition of standard dialysis fluids, may impair in vivo local defence, especially during the initial phase after dialysate inflow.

Table 1. Peritoneal Macrophage Function in CAPD Patients.

Parameter Measured	Finding	Comparative Cells
Maturation level	↓*	Normal Peritoneal macrophages
	↑*	CAPD peripheral monocytes
Phagocytic capacity	Normal	Normal peritoneal macrophages
	Normal	Normal PMN
	↓	CAPD peripheral monocytes
Bactericidal ability	Normal	Normal PMN
	Normal	Normal blood monocytes & peritoneal macrophages
	↓	Normal peritoneal macrophages
Oxidative metabolism		
-H_2O_2 generation	Normal	Normal peritoneal macrophages
-Chemiluminescence	↓	Normal peripheral or peritoneal PMN
	↑	Normal peritoneal macrophages
	↑	CAPD peripheral monocytes
Receptors		
-C5a	↑	Normal and CAPD peripheral monocytes
-Complement receptors CR1 and CR3	Normal	Normal and CAPD peripheral monocytes
-Fc receptors	↑	Normal and CAPD peripheral monocytes
	↑	CAPD peripheral monocytes

From reference 66.

*↓ = decreased.
*↑ = increased.

3.3. Mediators and messengers

3.3.1. Introduction

Macrophages are capable of producing a wide variety of substances, many of which establish communication with other cells. Substances, acting on the secreting cell (macrophage) itself, i.e. in an autocrine fashion or on other cells in the micro environment, i.e. in a paracrine fashion are referred to as local hormones or autacoids (91). When substances gain access to the circulation and are not rapidly destroyed, they may affect the function of distant organs, thereby displaying endocrine actions. Conversely, many substances can act on macrophages (2). Numerous substances such as prostaglandins and proteins interact with specific cell surface receptors. Interaction of the ligand (signalling molecule) with the cell surface receptor at the cell exterior induces the generation of an intracellular signal, finally leading to a biological effect. The intracellular molecules involved in the propagation of the signal are referred to as second messengers. In this chapter the properties of the prostaglandins, of the polypeptide interleukin-1, the polypeptide Tumor Necrosis Factor and of the second messenger cyclic AMP are discussed.

3.3.2. Eicosanoids: Prostaglandins and Leukotrienes

Arachidonic acid metabolites, collectively referred to as eicosanoids, are among the most important mediators and modulators of inflammation. The oxidative conversion of the lipophilic membrane bound fatty acid arachidonic acid gives rise to a variety of highly reactive compounds of intermediate lipid-water solubility. This increase in solubility in water allows the eicosanoids to interact with a variety of substances. Arachidonic acid is principally converted to either prostaglandins and thromboxanes by the endoplasmic reticulum bound enzyme cyclooxygenase or to the leukotrienes by the cytosolic enzyme 5-lipoxygenase (91). Eicosanoids are secreted immediately after their biosynthesis and are rapidly inactivated, implying that they exert their effects only locally (autacoids). They are thought to elicit a biological response following binding to specific cell surface receptors.

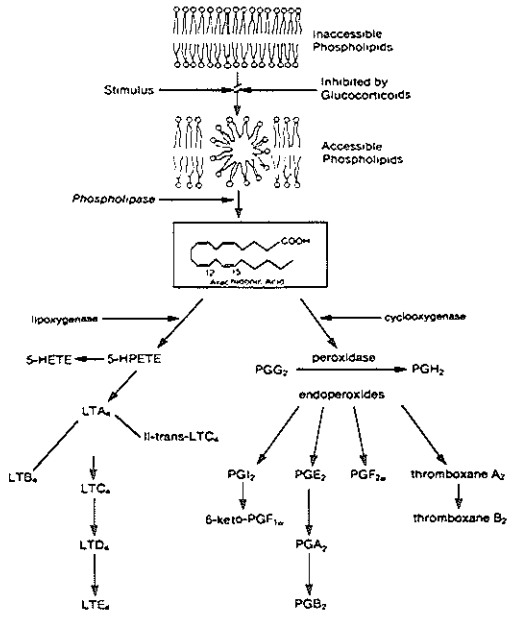

Fig. 2A Major products of arachidonic acid cascade.

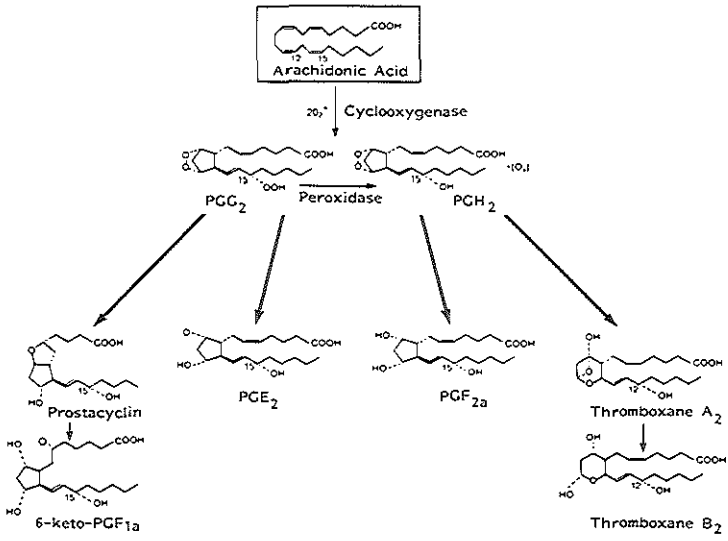

Fig. 2B Major cyclooxygenase products of macrophages (adapted from reference 78).

Mechanical or biochemical perturbation of the cell membrane induces liberation of arachidonic acid from the 2'-acyl position of cell membrane phospholipids, via activation of the membrane bound enzyme phospholipase A_2 (91). The release of arachidonic acid is considered to be the initial and rate limiting step in eicosanoids biosynthesis (78,79) Enzymatic oxidation of arachidonic acid by cyclooxygenase results in the formation of the unstable endoperoxides PGG_2 and PGH_2. Subsequently these intermediate compounds are enzymatically converted to the prostaglandins and thromboxanes. (The term prostanoids includes both prostaglandins and thromboxanes). The generation of the major metabolites in the arachidonic acid cascade is schematically presented in figure 2. The enzyme cyclooxygenase occurs in virtually every mammalian tissue, but subsequent steps in arachidonic acid metabolism differ in different cells. The fact that each cell type displays a characteristic profile of cyclooxygenase products, is of functional importance since these compounds have a broad spectrum of different or even opposing biological activities. The cyclooxygenase metabolites produced by mononuclear phagocytes are prostaglandin E_2 (PGE_2), prostacyclin (PGI_2), thromboxane A_2 (TXA_2) and Prostaglandin $F_{2\alpha}$ (89). It is reported that only slight amounts, if any, of PGI_2 are produced by human monocytes (89). Interestingly, human peritoneal macrophages were found to release PGI_2 in amounts comparable to PGE_2 and TXA_2 (70, chapter 6). The leukotrienes, generated by the lipoxygenase pathway of the arachidonic acid cascade, via the intermediate 5-HPETE, are principally produced by leucocytes (i.e. mononuclear phagocytes, mast cells, neutrophilic and eosinophilic granulocytes). Human monocytes and macrophages produce leukotriene B_4 (LTB_4) and leukotriene C_4 (LTC_4) (89). In conclusion, prostaglandins are highly reactive compounds that are secreted immediately after their synthesis in the tissues and are rapidly degraded. These properties allow a highly selective and versatile regulation of inflammatory and immune responses by prostaglandins (and other eicosanoids).

3.3.2.1. Mechanisms of action

The various arachidonic acid metabolites exert their biological effects in different ways. Here, the mode of action of PGE_2 and PGI_2 is discussed. These prostaglandins bind to specific, stimulatory, receptors on the cell surface, resulting in activation of the membrane bound adenylate cyclase via interaction of the prostaglandin-receptor complex with the stimulatory

G protein. G proteins are a class of proteins that couple activation of receptors (e.g. adrenoreceptors, prostaglandin receptors) to either activation or inhibition of target enzymes. Adenylate cyclase catalyses the conversion of ATP into cyclic AMP (cAMP), that subsequently binds to the regulatory subunits of a cAMP dependent protein kinase (protein kinase A). This results in activation of the catalytic subunits of protein kinase A due to dissociation of the holoenzyme into catalytic subunits and the regulatory subunit-cAMP complexes (54). Protein kinases are a class of enzymes, which catalyze the phosphorylation of proteins, including enzymes, thereby affecting their activity. In various cells, cAMP interacts with Ca^{2+}, another important second messenger, in several ways. For instance, cAMP dependent proteinkinase enhances Ca^{2+} influx in organelles, probably by phosphorylation of Ca^{2+} ion channels or pumps. Conversely, Ca^{2+} activates adenylate cyclase and enhances phospholipase A_2-activity resulting in stimulation of production of eicosanoids, including prostaglandins. It is reported that another mechanism by which cAMP may convey its biological effects on the genomic level, is by binding of the protein kinase regulatory subunit-cAMP complex to DNA, thereby altering gene transcription (54).

3.3.2.2. Biological properties of prostaglandins

Prostaglandins exhibit in different tissues a wide variety of properties. Moreover, each prostaglandin can elicit different biological responses in a given tissue (78,79). In order to create order from the bewildering variety of actions of prostaglandins, a classification of prostaglandin receptors was recently proposed. On the basis of studies with agonists and antagonists it was proposed, that receptors exist for each of the natural prostanoids, i.e. PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 (23). Furthermore, evidence was found for the existence of three subtypes of PGE_2 sensitive receptors in smooth muscle, referred to as EP_1, EP_2 and EP_3 (24). This classification could explain, for example, the dual effects of PGE_2 in guinea pig trachea: Interaction of PGE_2 with EP_1 and EP_2 causes contraction and relaxation respectively.

Prostaglandins are well known for their proinflammatory effects such as induction of the classical signs of inflammation: redness, swelling, heat and pain. These signs are largely due to the ability of prostaglandins to dilate vessels and increase vascular permeability. However, during the last decade, evidence has accumulated, that prostaglandins have a number of

inhibitory effects on inflammatory cells, such as macrophages, lymphocytes and neutrophils (14, 78, 86). E-type prostaglandins are found to have in vitro suppressive effects on several macrophage functions such as release of lysosomal hydrolase, locomotion, phagocytosis and shape change. These effects turned out to mediated via an increase in intracellular cAMP levels, although PGE might also have a direct effect on macrophage membranes (14). Similarly, PGI_2 is able to raise cAMP levels and to inhibit macrophage functions. The inhibitory effects of PGE_2 and PGI_2 could also be demonstrated in vivo on granulomatous inflammatory reactions where macrophages are the dominating cells. Furthermore, it was shown that macrophages isolated at a stage of the granulomatous inflammation, when the inhibitory effects of PGE_2 were maximal in vivo, displayed also the largest increase in cAMP, after stimulation with PGE_2 in vitro. Both the granuloma reducing effect of PGE in vivo and the PGE induced cAMP rise in vitro were counteracted by an adenylate cyclase inhibitor, corroborating the concept, that the anti inflammatory effects of PGE both in vivo and in vitro are mediated via stimulation of the adenylate cyclase complex (14). Since macrophages themselves secrete prostaglandins, a feedback loop controlling macrophage responses can be postulated. The downregulating effects of prostaglandins are also relevant to macrophage-lymphocyte interactions, which play a key role in the specific branch of the immune system. Thus, PGE_2 inhibits the expression of Ia antigens by macrophages and thereby downregulates antigen presentation (78). Macrophage functions can be suppressed also indirectly, for example via a PGE_2 induced decrease in the synthesis of macrophage activating products by lymphocytes (lymphokines). Occasionally, PGE_2 can have stimulatory effects on macrophages, for instance on endotoxin stimulated collagenase production (102). Moreover, PGE were found to exert either proinflammatory or anti-inflammatory actions on the tissue component of inflammation, dependent on the stage of the inflammatory reactions (14). These findings demonstrate, that also with the cellular component of inflammation, prostaglandins can play a dual role. This does not alter the fact however, that prostaglandins have predominantly a dampening effect on macrophage responses by raising cAMP via activation of the membrane bound adenylate cyclase. In contrast, the leukotrienes LTB_4 and LTC_4, 5-lipoxygenase metabolites of the arachidonic acid cascade, are thought to exert primarily stimulatory effects on leucocytes including macrophages thereby counterbalancing the effects of PGE_2 and PGI_2 (94). In particular,

leukotrienes are known as potent chemotactic compounds, contributing to cellular infiltration during inflammation.

3.3.3. Interleukin-1

3.3.3.1. History

In 1971 and 1972 Gery et al. reported that human leucocytes, probably adherent monocytes, release a factor, which in combination with non specific stimulants, is capable to induce proliferation of murine T-lymphocytes (37,38,39). This factor was named as lymphocyte activating factor (LAF). In 1979 LAF and a number of other factors, previously described as MP (mitogenic protein), HP-1 (helper peak-1), TRF-III (T-cell replacing factor III), TRF_m (T-cell replacing factor M_o), BAF (B-cell activating factor and BDF (B cell differentiation factor), were renamed as interleukin-1, in view of the fact, that these monokine activities, acting as communication signals between leucocytes, turned out to be actually different facets of a single biochemical entity (1). In the same period LAF was reported to be identical with the host derived fever-inducing factor, endogenous pyrogen (EP) (77) and with Leucocyte Endogenous Mediator (LEM), which induces neutrophilia, hepatic acute phase protein synthesis and decreased plasma iron and zinc levels (55,98). Since that time, a number of other biological activities proved to be associated with interleukin-1 (IL-1), including inducing of PGE_2 and collagenase by synovial cells, resorption of bone (42), and destruction of cartilage matrix, wasting of muscles (5), tumoricidal (61) and antiviral activity. Table 2A,B summarizes some of the in vitro and in vivo effects of IL-1 (95).

Table 2A. In vitro effects of IL-1.

Cell growth	
	Fibroblasts, keratinocytes, mesangial cells, glial cells, T cells, B cells, hematopoietic precursors
Cytotoxic effects	
	Cytotoxic for tumor cells, ß-islet cells, thyrocytes
Immunological effects	
	Induction of lymphocyte-activating cytokines
	Activation of natural killer cells in synergism with IL-2 and interferons
	Increased IL-2 receptors
	Chemotaxis of B cells and T cells
	Antibody production by B cells
	Increased macrophage cytotoxicity
	Activation of T- and B-lymphocytes
Inflammatory effects	
	Induction of collagen and procollagenase synthesis
	Bone resorption
	Induction of PGE_2 in fibroblasts, macrophages and endothelial cells
	Basophil histamine release
	Eosinophil degranulation
	Thromboxane release by neutrophils and monocytes
Effects on vascular tissue	
	Proliferation of vascular smooth muscle cells
	Induction of endothelial plasminogen-activator inhibitors
	Induction of procoagulant activity in endothelial cells
	Increased endothelial adhesiveness
Effect on other cytokines	
	Induction of IL-1, IL-2, IL-3, IL-6, TNF, IFN-γ, IFN-ß

From reference 95.

Table 2B. In vivo effects of IL-1.

Central nervous system
Fever
Brain PGE_2 synthesis
Increased slow-wave sleep
Increased adrenocorticotropin production
Decreased appetite
Haematologic effects
Neutrophilia
Lymphopenia
Stimulation of haematopoietic progenitors
Radioprotection
Increased CSF production
Metabolic effect
Increased acute-phase proteins
Decreased albumin synthesis
Inhibition of lipoprotein lipase
Hypo zincaemia, hypo ferremia
Increased insulin production
Decreased cytochrome P450
Increased sodium excretion
Vascular system
Hypotension and shock
PGE_2 and PGI_2 production
Increased leucocyte adherence
Decreased vascular resistance
Capillary leak syndrome

From reference 95.

3.3.3.2. Secretion

Since 1984, two molecular forms of IL-1 have been described, IL-1α and IL-1ß (67). From human monocytes, first IL-1ß was cloned and then IL-1α (71). Although human IL-1α and IL-1ß show only 25% homology for aminoacid residues, they have the same biological activities (95). First, IL-1α and IL-1ß are synthesized as 31 kDa precursor molecules, that are processed to the mature biologically active 17 kDa forms. Both the 31 kDa and 17 kDa forms are secreted, but no 17 kDa IL-1 is found intracellularly (4,48). There is no evidence, that extracellular 31 kDa IL-1 is processed to the mature 17 kDa form (48). The precursor form of IL-1α appears to be biologically active, whereas precursor IL-1ß is not. 11 kDa, 4 kDa and 2 kDa IL-1 fragments have been reported. These forms may have biological activity (25,27) Recombinant IL-1 proved to exert the biological activities, previously claimed for IL-1, with the exception of the effects on neutrophils, such as chemotaxis, degranulation and oxidative metabolism (85). At present it is unknown how IL-1 is secreted by the cell. Since IL-1 lacks a signal peptide and neither IL-1α nor IL-1ß are glycosylated, secretion through the Golgi apparatus seems unlikely. It is suggested that processing activity may be a cytosolic or peripheral membrane protein (48, 58).
Evidence suggests that processing of precursor IL-1ß closely precedes, or is coupled, to secretion of mature IL-1ß (48,58). On the other hand, it is also reported that IL-1 is released from the cell via a nonspecific pathway (50).

3.3.3.3. Cell sources

Although mononuclear phagocytes are the main source of IL-1, a number of other cells can be induced to release IL-1 in vitro, including dendritic cells, large granular lymphocytes, astrocytes, microglial cells, keratinocytes, endothelial cells, mesangial cells, corneal epithelium, fibroblasts and Langerhans cells and polymorphonuclear leucocytes (68,95). A variety of agents can induce IL-1 production such as complement components, immune complexes, plant lectins and other cytokines (95). Especially microbial wall components such as LPS (lipopolysaccharide) from gram negative bacteria, muramyl dipeptide (gram positive bacteria), Staphylococcus aureus exotoxin and Nocardia rubra skeleton (52), are potent stimuli for IL-1 production.

3.3.3.4. Mechanisms of action

IL-1 is thought to exert its biological effects through binding to specific receptors on the cell surface, resulting in transmembrane signal transduction. Two structurally distinct IL-1 receptors have been described now, referred to as type I IL-1 receptor ($IL\text{-}1R_I$) and type II IL-1 receptor ($IL\text{-}1R_{II}$) respectively. The 80 KDa type I receptor is primarily found on T-cells, keratinocytes, endothelial cells, fibroblast, synovia cells and hepatocytes (28). The 68 kDa type II receptor is found on B-cells, PMN's and mononuclear phagocytes. The two receptor types have different binding properties for IL-1α and IL-1ß. Type I receptors display a higher binding capacity for IL-1α, while type II receptors have a greater affinity for IL-1ß (96). Little is known in which way occupancy of the receptor triggers cellular responses. Evidence suggests, that binding of IL-1 to the receptor may induce liberation of arachidonic acid by activation of phospholipase A_2 (26). It is also suggested that IL-1 induces hydrolysis of the membrane phospholipid phosphatidylcholine, which may be converted to diaglycerol, resulting in the formation of arachidonic acid by the action of the enzyme diaglycerol lipase (26). Anyway, IL-1 is found to induce, both in vitro and in vivo, synthesis of prostaglandins, especially PGE_2 and leukotrienes (32). Furthermore, several biological effects of IL-1 are inhibited or blocked by the cyclooxygenase inhibitor indomethacin, pointing to the role of prostaglandins in IL-1 induced responses (25,27). Signal transduction via IL-1 induced phosphatidylcholine hydrolysis and diaglycerol liberation would allow also increased protein kinase C activation.

3.3.3.5. Modulation of production and activity

In view of the potentially injurious effects of IL-1, its actions are likely to be highly controlled. Theoretically this could be achieved by inhibition of IL-1 synthesis or, after synthesis of IL-1, by inhibition of its interactions with target cells. It appears that physiological control of IL-1 occurs by both mechanisms. Corticosteroids and prostaglandins appear to play an important role as native inhibitors of IL-1 synthesis by mononuclear phagocytes. Corticosteroids were found to suppress biosynthesis of IL-1α and IL-1ß at the transcriptional level (64,65). Furthermore, IL-1 stimulates adrenocorticotropic hormone (ACTH), which in turn stimulates corticosteroids. Probably the pituitary-adrenal activity is affected via IL-1

induced increase in corticotropin releasing factor (CRF).(11) These findings indicate the existence of a feedback circuit between IL-1 and the neuroendocrine system emphasizing that IL-1 can act as a classical endocrine hormone. It is generally assumed, that PGE_2 posttranscriptionally inhibits IL-1 secretion from monocytes-macrophages (25,27). This assumption is based on the finding that exogenous PGE_2 induced suppression of IL-1 secretion, as measured by the thymocyte proliferation assay, without a concomitant decrease in IL-1 mRNA (57,60). Since other cAMP elevating substances exerted similar effects, PGE_2 was thought to exert its inhibitory actions on IL-1 secretion via activation of adenylate cyclase (57). The prevailing opinion that IL-1 production is controlled by PGE_2 is mainly founded on these studies. However, in other studies, the PGE_2 induced suppression on thymocyte proliferation could be attributed to a direct effect on the assay system rather than to decreased IL-1 secretion (47,88). The question whether PGE_2 regulates IL-1 secretion by human peritoneal macrophages, is an issue of this thesis. As described in chapter 8, we conclude that PGE_2, contrary to the conventional view, is not a (universal) regulator of IL-1ß release from macrophages.

In recent years, various native substances, that antagonize the interactions of IL-1 with cellular targets, have been identified. The IL-1 inhibitory properties of most of these substances, derived from a variety of sources, were demonstrated through their ability to inhibit the IL-1 induced enhancement of thymocyte proliferation in the thymocyte proliferation assay (62). Recently, an IL-1 inhibitor secreted by human monocytes has been purified and could be characterized as a pure receptor antagonist (IL-1ra) (45). Likewise, a human monocytic cell line produced an IL-1 receptor antagonist (IRAP) (21). Both IL-1 receptor antagonists (IL-1ra and IRAP) seem to be identical (21). Recombinant IL-1ra/IRAP antagonizes IL-1α and IL-1ß actions both in vitro and in vivo (30,45). It is probably identical to an IL-1 inhibitor previously described in the urine of febrile patients and of patients with myelomonocytic leukaemia. Initially IL-1ra/IRAP was reported to bind selectively to IL-1 receptor type I, but recently it was found, that IL-1 ra/IRAP recognizes and blocks also binding to IL-1 receptor type II. (43,103). The fact that IL-1 ra/IRAP is produced by the same cells that produce IL-1, lends support to the view that this native antagonist functions as a negative feedback inhibitor, controlling certain IL-1 actions. In this connection it is noteworthy that IL-1 ra causes a striking reduction in mortality form endotoxin-induced shock (81).

A number of other substances that antagonize IL-1 activity, have been identified. For example, it is suggested that transforming growth factor ß, derived from monocytes and thrombocytes, may inhibit IL-1 actions by interfering with events distal to the interactions of IL-1 with its receptor (62). Most of the IL-1 inhibitors are only partially characterized. Further characterization in molecular terms could allow to elucidate the role of these native inhibitors in physiology and pathophysiology.

3.3.4. Tumor Necrosis Factor

3.3.4.1. History

Nearly hundred years ago, William Coley, a surgeon from New York, described regression of a tumor in a patient who developed an intercurrent infection (erysipelas) (3,83,93). Subsequently he attempted to treat cancer patients by administering them broths derived from cultures with live bacteria and at a later stage vaccines with killed bacteria. In some cases tumor necrosis was induced, but the results were inconsistent and serious complications often occurred. Studies by Coley's daughter Helen demonstrated that, especially, gram negative bacteria could cause haemorrhagic necrosis of mouse tumors. In 1943 Murray Shear et al (84). identified and purified the active component of the gram negative bacteria, a complex fat-and-sugar compound. This proved to be a constituent of the bacteria's outer wall and is now called lipopolysaccharide (LPS), or endotoxin. In the late 1950's Elisabeth Carswell, Lloyd Old and others demonstrated that another bacterial agent, Bacillus Calmette-Guérin (BCG) in combination with LPS induced the host (mouse) to produce a factor that was responsible for tumor killing (20). They called this factor Tumor Necrosis Factor (TNF), which was subsequently found to reproduce many of the effects of BCG and LPS. Carswell et al. also suggested that macrophages were the source of TNF. Eventually a single protein was obtained from the blood of mice and rabbits to which both in vitro and in vivo effects of TNF could be attributed. Carswell et al. proved that macrophages and other (human) cell types as well secreted TNF or TNF-like factors. In 1984 the TNF encoding gene was cloned, followed by the identification of the amino acid sequence and the production of large amounts of recombinant TNF (90). Since then, the TNF encoding genes proved to be very similar in a variety of species, while recombinant TNF could induce multiple biological responses (100). For instance, TNF has a

wasting effect, previously ascribed to a factor referred to as cachetin, which is caused by suppression of lipoproteinlipase and other enzymes required for triglyceride synthesis (12). Lymphotoxin, a protein produced by T-lymphocytes and B lymphoblastoid cell lines, and the macrophage derived TNF have almost all their bioactivities in common (3). On the molecular level, both substances share approximately 30% homology. The macrophage and lymphocyte product are referred to as TNFα and TNFß respectively.

3.3.4.2. Cell sources and biological responses

Although other cell types including Natural Killer cells and mast cells can produce TNF, mononuclear phagocytes are considered to be the main source of TNFα (12). A remarkable array of biological effects are described for TNF, exhibiting an almost complete overlap with those of IL-1 (see table 3) (85). The nature of many of these biological responses indicate that TNFα, like IL-1, is involved in inflammation and immunity. Conceivably, TNFα constitutes a link between the inflammatory response and defence against tumors (13). Like IL-1, TNFα exerts its effects in an autocrine, paracrine and endocrine fashion as well. By travelling via the circulation to virtually every part of the body, the inflammatory reactions can be orchestrated. A number of factors, especially microbial wall components such as LPS, toxic shock syndrome toxin and mycobacterial cord factor, are able to induce TNFα biosynthesis. Moreover, several host derived factors, such as C_{5a}, interferon γ, IL-1, granulocyte/macrophage colony-stimulating factor and TNFα itself can induce or enhance TNFα production and release (100).

Table 3. Comparison of target cells and actions of IL-1 and TNF/LT.

Target cells or tissues	Effects	IL-α,ß	TNF/LT
T lymphocytes	Enhances IL-2 receptor expression	+	+
	↑Lymphokine production	+	+
	Thymocyte comitogen	+	±
B lymphocytes	Enhances antibody production	+	+
	Promotes B cell proliferation	+	+
Hypothalamus/CNS	Release CRF → pituitary ACTH	+	-
	Induces prostaglandin mediated fever	+	+
	Induces somnolence and anorexia	+	+
Neutrophils	Chemoattractant	-	±
	Increases adhesiveness, phagocytosis, ADCC, and oxygen intermediates	-	±
	Degranulates → enzyme release	-	±
Monocytes/macrophages	Chemoattractant	-	±
	Induces production of prostaglandins, IL-1, IL-6, GM-CSF and NAP-1	+	+
	Coactivates to cytocidal state	+	+
Endothelial cells and vascular smooth muscle	Increases adhesiveness (ICAM 1)	+	+
	Induces procoagulant activity, IL-1, IL-6, GM-CSF, PAI, prostaglandins and MHC	+	+
	Mitogenic and angiogenic	+	+
Osteoblasts	Decreased alkaline phosphatase	+	+
Osteoclasts	Bone resorption (↑ collagenase)	+	+
Chondrocytes	Increased cartilage turnover	+	+
Fibroblasts/synovial cells	Mitogenic and express oncogenes	+	+
	Induces prostaglandins, IL-1, IL-6, collagenase and GM-CSF	+	+
Hepatocytes	Induces some acute phase reactants	+	+
	Decrease P450 and increased C3	+	+
	↑ Plasma Cu ↓ Fe + Zn	+	+
Bone marrow haematopoietic cells	Inhibits some precursor cell proliferation and differentiation	-	+
	Stimulates precursor cells	+	-
Tumor cells/virus infected cells	Selectively cytostatic/cytocidal	+	+
Adipocytes	Decreased lipoprotein lipase	±	+
	Increased lipolysis	±	+
Epithelial cells	Mitogenic	+	?
	Secrete collagen type IV	+	?
Pancreatic ß cells	Modulates insulin levels	+	-
Dendritic cells	Increased T cell activation	+	?

From reference 85.

3.3.4.3. Production and release

It is generally assumed, that the biosynthesis of TNFα begins with the elaboration of a signal peptide consisting of 76 amino acid residues, into the rough endoplasmic reticulum (59). After cleavage of the signal peptide, the elongation of 17 kDa polypeptide, consisting of 157 amino acid residues, follows which is rapidly secreted. Only very slight amounts of TNFα, if any, are found within the cell either in its 26 kDA or mature 17 kDa form (105), while outside the cell exclusively the mature 17 kDa form is found (19). It is claimed, that some of the 26 kDa TNFα may be present within the cell as a membrane associated protein, which may be relevant to paracrine biological activities (59). Thus it appears, that TNFα and IL-1 differ considerably in their way of synthesis and secretion (19,48). The regulation of TNFα release, especially the role of prostaglandins is discussed in the chapters 7 and 8. In this connection it is worth mentioning, that TNFα is found to stimulate release of adrenocorticotropic hormone (ACTH) in vitro (75). Therefore, it is conceivable, that in vivo TNFα release is controlled via ACTH induced secretion of corticosteroids.

3.3.4.4. Mechanisms of action

Specific receptors for TNF have been identified at the cell surface. Recently two immunologically distinct proteins which bind TNF and block its effects, were isolated from human urine (31). These two TNF binding proteins, which are likely derived from the cell surface receptors by proteolytic cleavage, could also detected in serum in increased amounts during inflammation (31). Little is known about the mechanism by which the TNF signal is transduced, but it has been shown that after binding of TNF to the cell surface it is internalized and degraded. In view of the fact, that 1) TNF induces PGE_2 release, 2) PGE_2 potentiates while 3) cyclooxygenase inhibitors suppress certain effects of TNF, PGE_2 appears to be involved in the regulation of TNF induced actions (63).

3.3.4.5. Modulation of production and activity of TNFα

In view of the potentially harmful effects of TNFα, its actions, like those of IL-1, must be controlled, either by regulation of its synthesis or its actions on target cells. As already mentioned, TNF release may be controlled via ACTH induced secretion of corticosteroids. The role of

prostaglandins in the regulation of TNFα release is an issue of this thesis. As described in chapter 8, we conclude, that PGE_2 controls TNFα release from macrophages. Recently, the interesting discovery was made that the actions of TNF on target cells may be regulated by naturally occurring inhibitors. Wallach et al. isolated from human urine two TNF binding proteins (31). Evidence suggests, that these proteins are soluble forms of two molecular species of TNF cell surface receptors. The soluble forms may be produced by proteolytic cleavage or shedding of the membrane form. Alternatively, the soluble and membrane forms may be coded by the same gene and synthesized independently by translation of different species of mRNA produced via alternative splicing pathways. Whatever the secretion mechanism may be, the existence of soluble TNF receptors raises the interesting possibility, that TNF receptors, if presented in soluble form to the cells, may sequester TNF thereby serving as a naturally occurring inhibitor for TNF actions.

3.3.4.6. IL-1 and TNF in disease

In view of the character of their activities, it is plausible that IL-1 and TNF play a crucial role in host defence mechanisms against infections. It was found indeed, that low dose recombinant IL-1 protects granulocytopenic mice from lethal gram negative infections (74). Recombinant TNF could protect mice against a normally lethal Listeria challenge (46). An increased local production of TNF was found in human tuberculosis (6). However, the putative role of IL-1 and especially TNF in the pathogenesis of septic shock (82,100), clearly demonstrates that these cytokines are two edged swords. Moreover, they may be involved in the pathogenesis of several diseases such as diabetes, rheumatoid arthritis and atherosclerosis (73). It implies, that IL-1 and TNF may be useful agents in the treatment of patients with severe infections.On the other hand, inhibition of their production or biological actions could be beneficial, in the event the damaging effects predominate.

3.3.4.7 Cytokine network

IL-1 and TNF have many regulating actions on cell functions in common. Furthermore, there are multiple interwoven interactions among these and other cytokines. Thus, IL-1 and TNF stimulate the production of each other and both IL-1 and TNF stimulate IL-6 production. TNF and IL-1 modulate

each other's cell surface receptors and act synergistically on various cell functions. In fact, IL-1, TNF, IL-6 and other cytokines appear to act together in a network. Cytokines can also suppress each other's production and actions: thus, IL-6 suppresses the production of IL-6 and IFN-γ decreases the IL-1 induced osteoclast activation. (95) Therefore, IL-1, TNF and other cytokines appear to constitute a complex network of interacting substances with positive and negative properties.

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Chapter 4

Measurement of Interleukin-1 and Tumor Necrosis Factor

4.1. Interleukin-1

Interleukin-1 (IL-1) was originally discovered as a factor derived from monocytes-macrophages, that has a co-mitogenic effects on T-lymphocytes (2). Hence, it was named lymphocyte activating factor (LAF). In 1979 LAF was renamed as interleukin-1 (IL-1), since LAF was found to be very similar or identical to a number of factors with different bioactivities. Until a few years ago, IL-1 was usually determined by measuring LAF-activity with the murine thymocyte proliferation assay. This assay is based on the ability of IL-1 to enhance in vitro proliferation of mouse thymocytes stimulated with a suboptimal dose of the plant lectins phytohaemagglutinin or concanavalin A. (2) The induced thymocyte-proliferation, as determined by incorporation of ^{3}H thymidine, is a measure for IL-1. The conventional view is that IL-1 enhances thymocyte proliferation by inducing the production of IL-2 and the expression of IL-2 receptors on thymocyte subpopulations. The main disadvantage of this assay is its lack of specificity. Thus, IL-6 synergizes with IL-1 to promote thymocyte proliferation (9). IL-2 and IL-4, when present in the test samples, induce thymocyte proliferation (5). Several products of macrophages have an inhibitory effect, such as PGE_2 (3) and Interleukin-1 receptor antagonist (11). In fact, thymocyte proliferation is the result of a series of complex interactions among different cells and cytokines. It may be very difficult to assess whether a substance such as PGE_2 has an effect on IL-1 production or on the assay system (10). During the last decade a number of bioassays for IL-1 have been developed using T-cell lines (1). In addition, assays using non-lymphical cells, such as endothelial or synovial cells, were developed. These assays fail to distinguish between IL-1 and TNFα (6). The lack of

specificity has been overcome, when immunoassays specific for human IL-1α and IL-1ß were developed (6), since human recombinant IL-1α and IL-1ß have become available (6). In our studies a commercially available enzyme linked immunosorbent assay (ELISA) for human IL-1ß has been employed. As described in chapter 5, the assay is carried out in 4 stages. Briefly, IL-1ß present in the test sample, will bind to monoclonal antibodies specific to human IL-1ß which are attached to microtitration wells (solid phase). In the second stage, polyclonal (rabbit) anti-IL-1ß will bind specifically to solid phase bound IL-1. In the third stage, added anti (rabbit) IgG conjugated to horse radish peroxidase will bind specifically to solid phase bound rabbit anti-IgG. In the fourth stage, added enzyme substrate will be oxidized by bound conjugate resulting in a coloured end product. The colour intensity is proportional to the amount of IL-1ß present in the sample. It should be noted that immunoassay may detect also the biologically inactive precursor IL-1ß. (4).

4.2. Tumor necrosis factorα (TNFα)

Tumor necrosis factor (TNF) was identified in 1975 as a factor capable to induce selective tumor necrosis. (7). Several assays for measurement of TNF have been developed based on the cytotoxic effects of TNF on specific mouse or rat cell lines. The mouse fibroblast like cell line L 929 is most commonly used for quantitative determinations of TNF by measuring the TNF induced cytolytic effects. Several substances, such as interferon-γ and IL-1 may interfere in L 929 cytotoxicity assays (7,8). In addition, different L 929 sublines show a variable sensitivity to TNF induced cytotoxicity. These factors affect thus the specificity and reproducibility of assays based on the determination of cytotoxic effects on L 929 cells (7). As described in chapter 7, in our studies TNFα-activity was quantified by determining the growth inhibitory (i.e. cytostatic) effects against L 929 target cells. Briefly, a suspension of L 929 cells is incubated in the presence of supernatant or medium alone as control. After 24 hours incubation, ^{3}H thymidine is added and the cell suspension is cultured for another 18 hours, whereafter ^{3}H thymidine uptake by target cells is measured. The percentage of growth inhibition (cytostasis) is calculated according to the formula:

$$\frac{CPM_{control} - CPM_{experimental}}{CPM_{control}} \times 100\%$$

A typical example of cytostatic effects of TNFα on L929 cells is shown in figure 1.

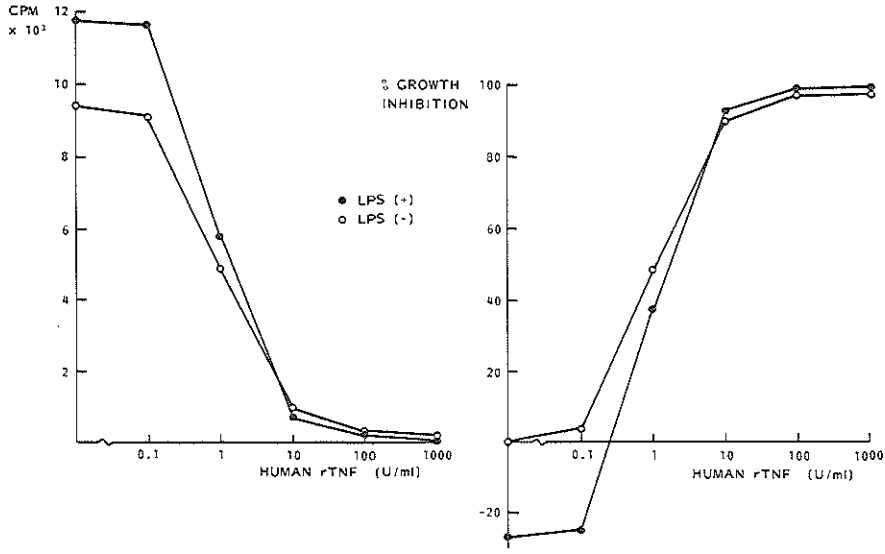

Fig. 1 A typical example of dose dependent effects of human recombinant TNFα on L 929 target cells in the presence (LPS+) or absence (LPS-) of 5 µg/ml/ of LPS. Proliferation of L 929 target cells is measured by ^{3}H thymidine incorporation (A) and the corresponding % growth inhibition calculated (see text) (B). Specific activity: $7x10^{7}$ U/mg human recombinant TNFα.

The cytostatic effects against target cells were neutralized by anti-TNFα antibodies, demonstrating that cytostasis was induced by TNFα. Moreover, human recombinant IL-1α had no additive effect on cytostasis. (see chapter 7). In the last few years, immunoassays for human TNFα have become available, allowing specific and reproducible determination of TNFα in biological samples (8). In our studies a commercially available two-site immunoradiometric assay (IRMA) was employed, as described in chapter 7. Briefly, monoclonal antibodies directed against distinct epitopes of human TNFα, are attached to the lower and inner surface of plastic tubes. After incubating the samples or standard in the coated tubes in the presence of I^{125} labelled antibodies to TNFα, TNFα is bound both to the labelled and coated antibodies. The radioactivity bound to the test tubes reflects TNFα contents.

In conclusion, immunoassays have the major advantage over bioassays in that they are specific and reproducible. However, as was found for TNF, immunoassays may detect denatured, aggregated and fragmented molecules, which may be biologically inactive (7). Furthermore, it should be taken into account that IL-1ß immunoassays may detect the biologically inactive IL-1ß precursor molecule (4), which is found not only within the cell, but also in the supernates.

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Chapter 5

Interleukin-1 release from peritoneal macrophages

(published paper)

Endotoxin-stimulated peritoneal macrophages obtained from Continuous Ambulatory Peritoneal Dialysis patients show an increased capacity to release interleukin-1ß in vitro during infectious peritonitis.

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ABSTRACT

Interleukin-1 (IL-1) release by peritoneal macrophages obtained from patients on Continuous Ambulatory Peritoneal Dialysis (CAPD) was studied in 9 patients during an infection-free period and 8 patients during an infectious peritonitis, using an ELISA for IL-1ß. Without exogenous stimulation with LPS, peritoneal macrophages from infected and uninfected patients released the same amounts of IL-1ß, 183 $\pm$ 40 pg ml^{-1} $(24h)^{-1}$ per 10^6 cells (means $\pm$ SEM) and 251 $\pm$ 96 pg ml^{-1}, respectively. However, in response to a dose of 5 µg ml^{-1} of LPS, peritoneal macrophages released significantly more ($p<0.005$) IL-1ß during peritonitis (6579 $\pm$ 2793 pg ml^{-1} $(24h)^{-1}$ per 10^6 cells) as compared to the infection-free period (1040 $\pm$ 182 pg ml^{-1}). These findings show, that after microbial invasion of the peritoneal cavity, peritoneal macrophages are primed in vivo to release an increased amount of IL-1ß in vitro after subsequent exogenous stimulation with LPS, indicating that peritoneal macrophage activation for IL-1ß secretion occurs in steps.

KEYWORDS

Interleukin-1; macrophages; peritoneal dialysis, continuous ambulatory (CAPD); peritonitis; infection.

INTRODUCTION

Peritoneal macrophages, obtained from effluent dialysate of patients on Continuous Ambulatory Peritoneal Dialysis (CAPD) produce in vitro various mediators such as cyclooxygenase products (1) as well as the second messenger cyclic AMP (cAMP) (2). These substances are supposed to have down regulatory effects on the activity of phagocytic cells (3). Previously we reported that peritoneal macrophages from patients on CAPD obtained during an infectious peritonitis display a diminution in the basal secretion of cyclooxygenase products and in the cAMP production (4). We postulated that the diminished production of these substances with down regulatory properties would enable the macrophages to secrete an increased amount of interleukin-1 (IL-1) (5), which is a key mediator of the host defence system during infection. The characteristics of IL-1 have

been reviewed recently (6). Two molecular forms of IL-1, IL-1α and IL-1ß respectively, are distinguished (7). IL-1α is mainly membrane bound whereas IL-1ß is secreted into the environment (6). In this way IL-1ß gains access to the circulation and can exert systemic effects, which allows orchestration of the inflammatory response. The aim of the present study was to investigate the in vitro release of IL-1ß by peritoneal macrophages of CAPD patients, both during an infection-free period and during peritonitis.

PATIENTS AND METHODS

Patients

Macrophages were obtained from effluent dialysate of patients undergoing CAPD because of end stage renal disease (8). Four times a day commercially available peritoneal dialysis solutions consisting of 132 mmol l^{-1} Na^{+}, 1.75 mmol l^{-1} Ca^{++}, 0.75 mmol l^{-1} Mg^{++}, 102 mmol l^{-1} Cl^{-} 35 mmol l^{-1} lactate and 1.36%, 2.27% or 3.86% anhydrous dextrose (DIANEAL^{R}, Baxter Laboratories Deerfield, Illinois, USA) were changed. Out of 11 patients taking part in the study (table 1), 6 patients were studied both during an infection-free period and during peritonitis, while 3 other patients participated only during an infection-free period and 2 patients only during an episode of peritonitis (table 2a, 2b).

Table 1. Characteristics of CAPD patients participating in the study

Patient no.	Sex	Age (years)	Primary renal disease
1	f	56	Diabetic nephropathy
2	m	71	Cholesterol emboli
3	m	65	Diabetic nephropathy
4	f	53	Haemolytic uraemic syndrome
5	m	77	Hypertensive nephropathy
6	m	59	Diabetic nephropathy
7	f	32	Chronic glomerulonepohritis
8	f	21	Diabetic nephropathy
9	f	49	Diabetic nephropathy
10	m	34	Chronic glomerulonephritis
11	f	51	Polycystic disease

Table 2. Data of nine CAPD patients during an infection-free period (a), and data of eight CAPD patients during peritonitis (b)

(a) Patient no.	Dialysate leucocyte count ($x10^6L^{-1}$) (%)	Macrophages after purification	Time interval between collections of dialysate and previous episode of peritonitis (months)	Time on CAPD until collection of dialysate (months)
1	9	86	2	4
2	8	83	8	10
3	21	80	7	19
4	10	81	*	2
5	1	95	15	19
6	20	98	*	4
7	3	89	3	37
8	1	95	2	20
9	8	100	8	38

(b)			Causative microorganism	
4	1200	86	Candida albicans	3
5	3600	87	Staphylococcus epidermidis	22
6	1800	97	Staphylococcus epidermidis	5
7	2400	93	Staphylococcus epidermidis	34
8	2100	88	Acinetobacter	22
9	300	83	Staphylococcus epidermidis	40
10	300	89	Streptococcus sanguis	24
11	200	96	Staphylococcus aureus	4

From all 8 patients who developed peritonitis from the start of the study, cells were harvested from one of the first bags in which opalescence appeared, prior to the intraperitoneal administration of antibiotics. The majority of peritonitis episodes was caused by gram positive cocci in accordance with the findings reported in literature (8). In 6 out of the 8 peritonitis patients cells were harvested from the morning bags.
The diagnosis was confirmed by an elevated white blood cell count (>100 mm^{-3}) in the effluent dialysate with over 50% of neutrophils at differential

counting and/or a positive microbial culture (8) Patients with a relapsed peritonitis or with peritoneal eosinophilia were excluded from the study. From the 9 patients studied during an infection-free period, cells were harvested from the morning dialysis bags. No patient was studied within 2 months of an episode of peritonitis or within two months after starting CAPD (table 2A, 2B). After drainage of the peritoneal dialysate the bags were kept at 4° C whereupon the cells were harvested within 24 hours.

Isolation of Peritoneal Macrophages

The cells were harvested from the dialysate by centrifugation (5 min. at 4°C, 700 g) and washed twice with Dulbecco's modified eagle's medium (DMEM) (Gibco Ltd, Paisley, Scotland). After this the cells were layered over a Percoll^{R} solution (Pharmacia LKB Biotechnology, Uppsala, Sweden) with a density of 1.064 g ml^{-1} (9) and centrifugated (30 min. at 4°C, 400 g). The resulting cell layer was removed and the cells were washed twice in DMEM. After resuspensation in RPMI 1640, supplemented with 10% foetal calf serum, 2 mmol l^{-1} glutamin, 100 IU ml^{-1} streptomycin-penicillin (all Flow Laboratories, Irvine, Scotland), 2 g l^{-1} $NaHCO_3$ (Merck, Darmstadt, FRG) and 5 x 10^{-5} mol l^{-1}ß-mercapto-ethanol (Merck, Schuchardt, FRG), the cells were counted in a Bürker chamber. Viability was determined by trypan blue exclusion and was always found to be higher than 95%. The cell suspensions were cytocentrifuged and the smears were stained with Hemacolor^{R} rapid blood smear staining (Merck, Darmstadt, FRG) and with non-specific esterase staining (10). After differential counting it was judged that 93 ± 7% of the Percoll-separated peritoneal cells from patients in a infection-free period were macrophages. The cells of the peritonitis patients were found to be 89 ± 5% macrophages (table 3). 1.0 ml of the 1.0 x10^{6} cells ml^{-1} suspension was placed in 35 mm diameter tissue culture well (Costar, Cambridge, MA, USA). The cells were cultured in a moist chamber at 37° C, 7.5% CO_2 for 24 hours for which period the cells were exposed either to 5 µg ml^{-1} of LPS (E. coli 0111:B4) (Sigma chemical company, St. Louis, MO, USA) or to the medium alone. After the culture period the supernatants were removed, filter sterilized over a 0.22 µm Millex^{R} filter (Millipore SA, Molsheim, France) and stored at -70°C.

Table 3. Peritoneal leucocyte differential counting before and after purification procedure

	Infection-free period (purification)		Peritonitis (purification)	
	Before (%)	After (%)	Before (%)	After
Macrophages	85 ± 10*	93 ± 7	25 ± 12	89 ± 5
Neutrophils	5 ± 3	0	75 ± 12	10 ± 5
Lymphocytes	10 ± 10	7 ± 7	0.25 ± 0.5	1 ± 2

* Values expressed as means ± SD

Interleukin-1ß Assay

IL-1ß concentrations of peritoneal macrophage supernatants were determined using a commercially available enzyme linked immunoabsorbent assay (ELISA) (Cistron Biotechnology, Pine Brook, NJ, USA), as described elsewhere (11). Briefly, supernatants or IL-1ß standard were added to microtiter wells coated with monoclonal antibodies specific to IL-1ß. After washing, polyclonal rabbit anti human IL-1ß was added to bind to solid phase bound IL-1ß. After the subsequent washing step, goat anti rabbit IgG, conjugated to horse radish peroxidase enzyme, is added. Finally, the chromogenic reaction, produced by added enzyme substrate, was measured, using a scanning spectophotometer. IL-1ß concentrations were obtained from a standard curve. The assay is sensitive to 20 pg ml^{-1}. In each ELISA-kit samples from both infected and uninfected patients were determined, in duplicate.

Statistics

Data were expressed as means ± standard error of the mean. Statistical significance was determined by Wilcoxon test.

RESULTS

In 7 out of 9 uninfected patients peritoneal macrophages released detectable amounts of immunoreactive IL-1ß (251 ± 96 pg ml^{-1}) without

exogenous stimulation with LPS (fig. 1). Below detection, an arbitrary value of 19 pg ml^{-1}, was assigned. After stimulation of these cells with a dose of 5 ug ml^{-1} of LPS a significant increase in IL-1ß release (1040 ± 182 pg ml^{-1}) was found ($P<0.005$). When macrophages were obtained during peritonitis, in 6 out of 8 patients the macrophages released detectable amounts of IL-1ß (183 ± 40 pg ml^{-1}). After stimulation with LPS, IL-1ß release of these cells increased significantly to values of 6579 ± 2793 pg ml^{-1} ($p<0.005$). When infected and uninfected patients were compared (fig.1) no difference in IL-1ß release by macrophages was found in the absence of exogenous LPS. On the other hand, endotoxin stimulated macrophages displayed significantly higher IL-1ß release during peritonitis as compared to the infection-free period ($p<0.005$).

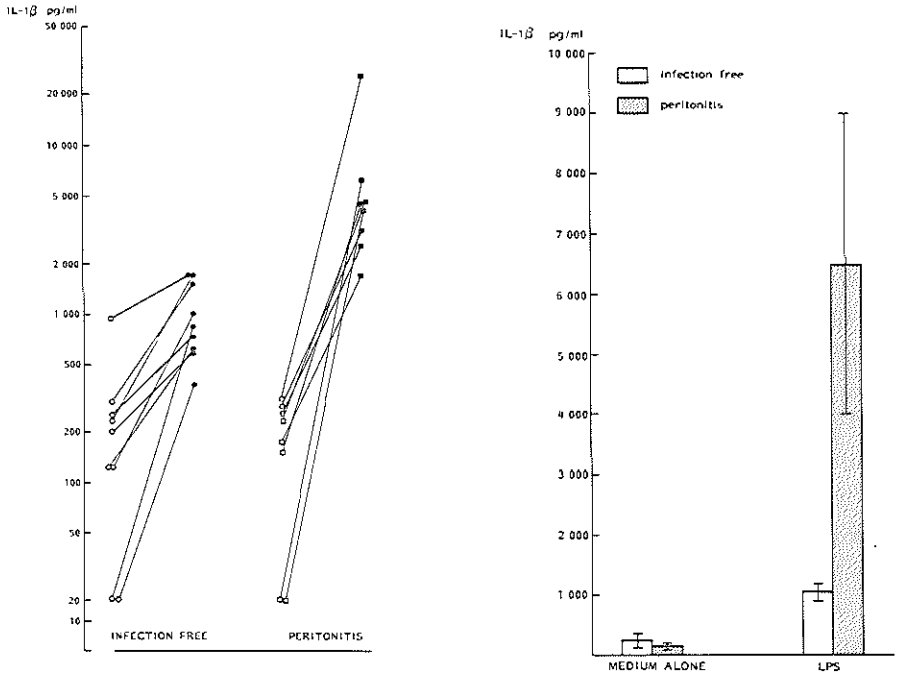

Fig. 1 Left panel: Comparison of IL-1ß release in vitro by peritoneal macrophages of CAPD patients either obtained during an infection-free period (○ ●) or during infectious peritonitis (□ ■). A line connects the data obtained, presented on a logarithmic scale, for each individual in the absence (○ □) or presence (● ■) of 5 µg of LPS ml^{-1} in culture medium. Right panel: Comparison of IL-1ß release in vitro (means ± SEM), presented on a linear scale, of peritoneal macrophages of CAPD patients either in the absence (left) or presence (right) of 5 µg of LPS ml^{-1} during an infection-free period (open bars) and peritonitis (hatched bars).

DISCUSSION

IL-1 is a pro-inflammatory polypeptide exerting its effects both locally and systemically. Mononuclear phagocytes are its main source. We studied IL-1 release of peritoneal macrophages obtained from effluent dialysate of CAPD patients and made a comparison between macrophages from an infection-free period and from peritonitis. Following inflow of fresh dialysate, white blood cells accumulate in the peritoneal cavity. During an infection-free period approximately 85% of the leukocytes as collected from effluent dialysate, are macrophages (1,12). Evidence suggests that these macrophages are blood-derived monocytes, not yet fully differentiated into macrophages (12). From studies on rodents, monocytes having entered local tissues, either develop to quiescent macrophages, or undergo activation following inflammatory stimuli (13). During peritonitis a rise of white blood cell count in the dialysate is found, composed of predominantly neutrophils and further mononuclear phagocytes, whereas lymphocytes are scant. Similarly, directly after intradermal injection of E.coli in mice, migration of neutrophils and monocytes from the vessels occurs simultaneously (14,15). Both the cells collected during peritonitis and during an infection-free period were subjected to the same separation procedure, whereupon equal pure macrophage populations were obtained. The present study shows, that peritoneal macrophages release increased amounts of IL-1ß, the main secretory form of IL-1, when stimulated with LPS. IL-1 has been demonstrated in macrophages of different origin (16,17,18). Until recently IL-1 secretion was determined by bioassay mainly using thymocytes. However, eicosanoids and interleukin-6 (19), may interfere with these assays. Recently, radioimmunoassay of IL-1ß has been published (20). In the present study macrophages stimulated with a high standard dose of LPS released in vitro higher amounts of Il-1ß, when they were obtained during peritonitis. On the other hand, unstimulated macrophages released the same amounts of IL-1ß, irrespective of they were obtained during peritonitis or during an infection-free period. Despite that the cells obtained during peritonitis may have been exposed during culture to phagocytic stimuli by intracellular microorganisms (21), additional stimulation is required to induce a higher release of IL-1ß as compared to the infection-free period. These findings are consistent with the concept, that macrophage activation occurs stepwise (22). Similarly, in order to obtain full tumoricidal activity, macrophages need to be primed by lymphokines, while actual secretion of lytic substances is triggered by LPS

(23). The concept of stepwise activation of macrophages appears to apply to the development of many functional responses (13). Chemotactic factors present in the peritoneal cavity during peritonitis may have recruited a different subpopulation of mononuclear phagocytes.
Once set in motion, the inflammatory response is regulated especially by the secretory products of mononuclear phagocytes (15). Previously we found that peritoneal macrophages of CAPD patients released a decreased amount of cyclooxygenase products during peritonitis (4). We presumed that the diminished release of these substances with down regulatory properties would be associated with an increased release of the pro-inflammatory polypeptide IL-1 (5). Now that we found an increased capacity to release IL-1ß during peritonitis following an appropriate stimulus in vitro, further experiments are required to analyse, whether cyclooxygenase products actually may affect IL-1ß secretion of peritoneal macrophages.
Arend e.a. (24) found, concerning human monocytes, that IL-1ß is present intracellulary to a greater or less extent, dependent on culture conditions, almost entirely in its biologycally inactive 32kDa precursor form. Because presently IL-1ß was determined only in supernatants, it cannot be decided whether the LPS induced enhanced IL-1ß secretion during peritonitis can be attributed to an increased synthesis, processing or exclusively to increased secretion of IL-1ß.
We did not determine IL-1 secretion in vivo. Nonetheless, an increase of IL-1 and/or related cytokines in vivo does not seem unlikely in view of the higher serum levels of the acute phase reactant C reactive protein, as found in CAPD patients during peritonitis (25). Furthermore, IL-1 may be involved in the pathogenesis of sclerosing peritonitis (26).

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Chapter 6

Release of Interleukin-1 and prostaglandins from peritoneal macrophages

(accepted paper)

Peritoneal Macrophages from Patients on Continuous Ambulatory Peritoneal Dialysis show a Differential Secretion of Prostanoids and Interleukin-1ß

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Prostaglandins, Leukotrienes and Essential Fatty Acids, (accepted).

ABSTRACT

In vitro secretion of the prostanoids PGE_2 and PGI_2 and of the cytokine IL-1ß by peritoneal macrophages obtained from CAPD patients during episodes of peritonitis and infection-free periods, was determined, after culturing with or without 5 µg/ml of LPS. The release of PGE_2 and PGI_2 as measured by its stable metabolite 6-keto-PGFα was determined in 10 episodes of peritonitis and 10 infection-free periods. IL-1ß release was determined in 14 episodes of peritonitis and 20 infection-free periods. PGI_2 release from macrophages declined sharply during peritonitis both in the absence and presence of LPS in the culture medium ($p < 0.005$). A tendency to decreased PGE_2 release was found during peritonitis, when macrophages were cultured in the absence of LPS. In the presence of LPS, the same amounts of PGE_2 were released during peritonitis and during an infection-free period. On the other hand, peritoneal macrophages released significantly more IL-1ß during peritonitis as compared to an infection-free period, provided that the cells were in vitro stimulated with LPS. In view of the interregulatory effects between prostanoids and macrophage cytokines in their production, these findings may indicate that the impaired release of PGI_2 during peritonitis has allowed the macrophages to secrete more IL-1ß after in vitro stimulation with LPS. This implies that PGI_2 and PGE_2 may play a distinct role in the regulation of cytokine secretion by these cells.

INTRODUCTION

Peritoneal macrophages collected from effluent dialysate of patients on Continuous Ambulatory Peritoneal Dialysis (CAPD) because of end stage renal failure produce various mediators *in vitro* (1,2). Recently we reported, that the secretion of the proinflammatory polypeptide interleukin-1ß (1L-1ß) by peritoneal macrophages is increased during an episode of infectious peritonitis, provided that these cells were stimulated *in vitro* with LPS (3). IL-1 is one of the key mediators of the host defence system during infection. IL-1ß is secreted into the environment and gains access to the circulation, thus allowing orchestration of the inflammatory response (4,5). We found also that the secretion of prostanoids and the production of the second messenger cyclic AP by peritoneal macrophages were decreased when CAPD was complicated by peritonitis (6). We suggested, that the

increased secretion of the proinflammatory IL-1ß during peritonitis could be linked to the decreased release of the prostanoids PGE_2 and PGI_2 (prostacyclin) (7) which are recognized to suppress macrophage functions (8), including the secretion of IL-1. In our previous studies on prostanoid release (6,7), the macrophages were not challenged with LPS, whereas we applied this stimulus for the study of IL-1ß secretion (3). In the present study we examined the relationship between the secretion of IL-1 and the prostanoids PGE_2 and PGI_2 in the same cultures under the uniform condition of endotoxin challenge. Peritoneal macrophages were collected during infection-free periods and episodes of peritonitis and cultured in presence of LPS. Several of the supernatant samples were also used for assessing their contents of both the prostanoids and the cytokine IL-1ß.

PATIENTS AND METHODS

Patients

A total number of 19 patients on CAPD participated in this study: 9 females and 10 males. The primary renal disease was diabetic nephropathy in 7 patients, hypertensive nephropathy (3 patients), chronic glomerulonephritis (2 patients), and one each case of cholesterol emboli, hemolytic uremic syndrome, chronic pyelonephritis, focal glomerulosclerosis, fibromuscular dysplasia and polycystic disease. In one case the primary renal disease was not characterised. In 14 episodes of peritonitis the causative microorganism was identified as a gram positive bacterium (10x), a gram negative bacterium (4x) and yeast (1x). (in 1 episode of peritonitis 2 different microorganisms were cultured). (table 1). Macrophages were collected from dialysate of CAPD-patients. Commercially available, dextrose containing dialysis solutions were used (Dianeal^R, Baxter Laboratories Deerfield, Illinois, USA). During 14 episodes of peritonitis observed in 11 patients, cells were harvested from one of the first bags with cloudy dialysate, prior to the administration of antibiotics. In 12 out of the 14 peritonitis episodes these were morning bags after overnight dwell. The diagnosis was confirmed by an elevated dialysate leukocyte count (>100/mm^3) consisting of over 50% polymorphonuclear cells (PMN) and/or a positive microbial culture (10). During 20 infection-free periods in 16 patients, cells were harvested from the morning bags. Eight patients participated in the study both during an infection-free period and an episode of peritonitis.

Table 1. Clinical data concerning the study on the secretion of prostanoids and interleukin-1ß by peritoneal macrophages collected from 19 CAPD patientens during 20 infection-free periods and 14 episodes of peritonitis.

	infection-free periods		episodes of peritonitis		
patient no	PG's	IL-1ß	Causative microorganism	PG's	IL-1ß
1	+)*	+	Candida albicans	+	+
	-)**	+			
2	-	+	Staphylococcus epidermidis	-	+
			Streptococcus viridans	+	+
3	+	+	Staphylococcus epidermidis	-	+
	+	+	Acinetobacter	+	+
4	+	+	Staphylococcus epidermidis	+	+
5	+	+	Acinetobacter	-	+
	+	+			
6	-	+	Staphylococcus epidermidis	+	+
			Streptococcus viridans	+	+
7	-	+	Enterococcus	-	+
8	-	+	Staphylococcus epidermidis and enterobacter	+	+
9			Streptococcus viridans	+	+
10			Staphylococcus aureus	+	+
11			Pseudomonas	+	+
12	+	+			
13	-	+			
14	-	+			
	-	+			
15	-	+			
16	+	+			
17	-	+			
18	+	+			
19	+	+			

PG's = prostanoids
IL-1ß = interleukin-1ß
)* PG's IL-1ß determined
)** PG's not determined

Prostanoids and IL-1ß were determined from the same macrophage cultures collected during 10 infection-free periods and 10 episodes of peritonitis. (table 1).
No patient was studied within 2 months of a peritonitis episode neither within 2 months after starting CAPD. After drainage of the dialysate the bags were kept at 4°C, whereupon the cells were harvested within 24 hours. None of the patients have taken cyclooxygenase inhibitors prior to the collection of dialysate.

Cell preparation

The peritoneal cells were harvested, isolated and subsequently cultured using methods described elsewhere (3). Briefly, after harvesting the cells from effluent dialysate, the macrophages were isolated by centrifugation through a Percoll^R solution. Subsequently, the cells were resuspended in RPMI 1640 supplemented with 10% fetal calf serum. According to differential counting of the cell smears stained with Hemacolor^R rapid blood smear staining and non specific esterase, the Percoll separated cell suspensions were composed of 93 ± 7% macrophages, 5 ± 5% lymphocytes and 2 ± 2% PMN's when obtained during an infection-free period, and of 89 ± 5% macrophages, 1 ± 1% lymphocytes and 10 ± 5% PMN's when obtained during peritonitis (values expressed as mean ± SD). One ml of 1.0 x 10^6 cells/ml suspension was placed in 35 mm diameter tissue culture wells and cultured at 37° C for 24 hours, in the presence of either 5 µg/ml of LPS or of the medium alone. After the culture period, the supernatants were stored at -70°C.

Assay for PGE_2 and PGI_2

The macrophage supernatants were applied to Sep Pak silica cartridges (Waters Associates, Etten-Leur, The Netherlands) and eluted with ethanol. The extracts were evaporated to dryness and dissolved in RIA buffer. Prostaglandin Standards were obtained from the Sigma Chemical Company (St. Louis, USA). Tritiated 6-keto-prostaglandin F1α or prostaglandin E2 (both from Amersham, UK) and antibody (both from Advanced Magnetics, Cambridge, USA) were added. After incubation, charcoal suspension was added. After 15 minutes at 4°C, the tubes were centrifugated and decanted.

Counts were measured in a beta-counter (Packard). The method was described in detail elsewhere (6).

Assay for interleukin-1ß

IL-1ß concentrations in peritoneal macrophage supernatants were determined using a commercially available enzyme linked immunoabsorbent assay (ELISA) (Cistron, Biotechnology, Pine Brook, NJ, USA) as described elsewhere (11,3) The assay is sensitive to 20 pg/ml. IL-1ß determinations were done in paralel duplicate samples from both infected and uninfected patients.

Statistics

Data were expressed as mean ± standard error of the mean. Statistical significance was determined by Wilcoxon test.

RESULTS

Secretion of prostaglandins

The release of prostacyclin (PGI_2) and PGE_2 was determined in the presence or absence of LPS in peritoneal macrophages collected during 10 infection-free periods and 10 episodes of peritonitis. The release of prostacyclin (PGI_2) (as determined by its stable metabolite 6-keto-PGF 1-α), by infection-free macrophages was 19,073 ± 3,616 pg/ml and 21,378 ± 4,275 pg/ml, in the absence or presence of 5 µg/ml of LPS respectively (figure 1A). A sharp decline in prostacyclin release from peritoneal macrophages obtained during an episode of peritonitis, was found both in the LPS stimulated (3,991 ± 1,194) and unstimulated cells (3,405 ± 1,442 pg/ml $p < 0.005$). Neither infection-free nor peritonitis macrophages displayed a significant increase in PGI_2-release in response to LPS. PGE_2 release of peritoneal macrophages obtained during an infection-free period was 15,100 ± 2,692 pg/ml, in the absence of LPS (figure 1B). Following stimulation with LPS, PGE_2-release of infection-free macrophages increased significantly to values of 27,405 ± 4,489 pg/ml ($p < 0.005$).

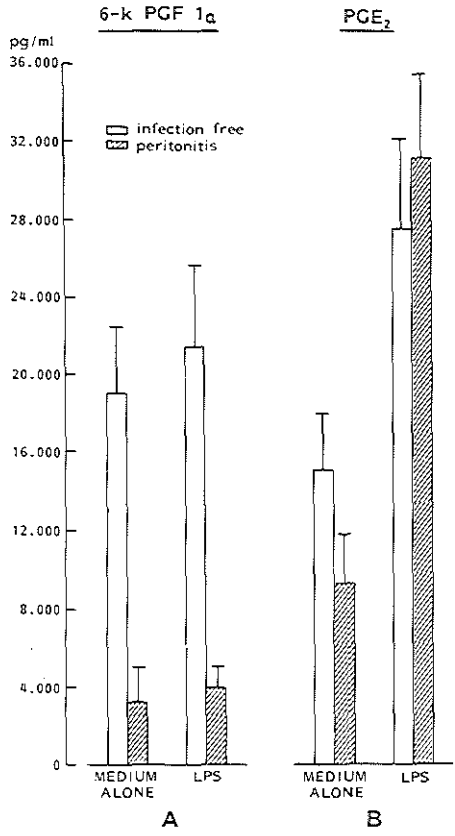

Fig.1 Release of PGI_2 (means ± SE), as determined by its stable metabolite 6-keto PGF 1-α (A), and of PGE_2 (B), in supernatants after 24 hours culture of peritoneal macrophages of CAPD patients either in the absence (left) of presence (right) of 5μg/ml of LPS, collected during an infection-free period (open bars) (n=10) or peritonitis (hatched bars) (n=10).

During peritonitis, a decrease, albeit not statistically significant, of PGE_2-release was found as compared to an infection-free period, when the macrophages were cultured in the absence of LPS, (9,424 ± 2,556 pg/ml). Similarly to the infection-free macrophages, peritonitis macrophages showed a significant increase in PGE_2-release in response to LPS (27,405 ± 4,489 and (31,007 ± 4,390 pg/ml respectively, $p < 0.005$). This difference in LPS stimulated PGE_2 release between infection-free and peritonitis macrophages was not significant.

IL-1ß secretion

The in vitro IL-1ß secretion by peritoneal macrophages from CAPD patients was determined in the presence or absence of LPS in 20 infection-free periods and 14 episodes of peritonitis. In the absence of LPS, peritoneal macrophages secreted detectable amounts of IL-1ß in 18 out of 20 infection-free periods (458 ±74 pg/ml). Below detection, a value of 19 pg/ml was assigned. In the presence of 5µg/ml of LPS, significantly more IL-1ß was released (1365 ±109pg/ml, $p < 0.005$). When macrophages were collected during peritonitis, a detectable release of IL-1ß was found in 12 out of 14 peritonitis episodes. (363 ± 122 pg/ml) after culture in the absence of LPS. Following exogenous stimulation with 5 µg/ml of LPS, in vitro IL-1ß release by peritonitis macrophages was significantly increased to values of 5935 ± 1629 pg/ml, ($p < 0.005$). Comparison of infection-free and peritonitis macrophages indicated that, no statistical difference in IL-1ß release is found, in the absence of LPS. However, LPS stimulated macrophages released significantly more IL-1ß during peritonitis as compared to an infection-free period ($p < 0.005$). As shown in table 2, IL-1ß release from macrophages of uninfected patients was similar, whether or not a previous episode of peritonitis occured. This applies both for basal and LPS stimulated IL-1ß release.

Figure 2 shows a typical example of mean IL-1ß release by peritoneal macrophages obtained during 3 infection-free periods and 3 peritonitis episodes, in response to graded doses of LPS. Both in the infection-free and the peritonitis macrophages, maximum IL-1ß release was attained by stimulation of LPS at a dose of 5µg/ml ($5x10^{-6}$g/ml). Peritonitis macrophages showed a definite dose dependant increase within the range of $5x10^{-6}$ - $5x10^{-9}$ g/ml of LPS.

Table 2.

IL-1ß release from infection-free patients.

	previous peritonitis (n=13)	no previous peritonitis (n=7)
without LPS	477 ± 132	420 ± 109
with LPS	1292 ± 218	1501 ± 202

IL-1ß release (pg/ml) from peritoneal macrophages collected during an infection-free period from patients with or without a previous episode of peritonitis. IL-1ß release from anyone group of patients did not show statistically significant differences.

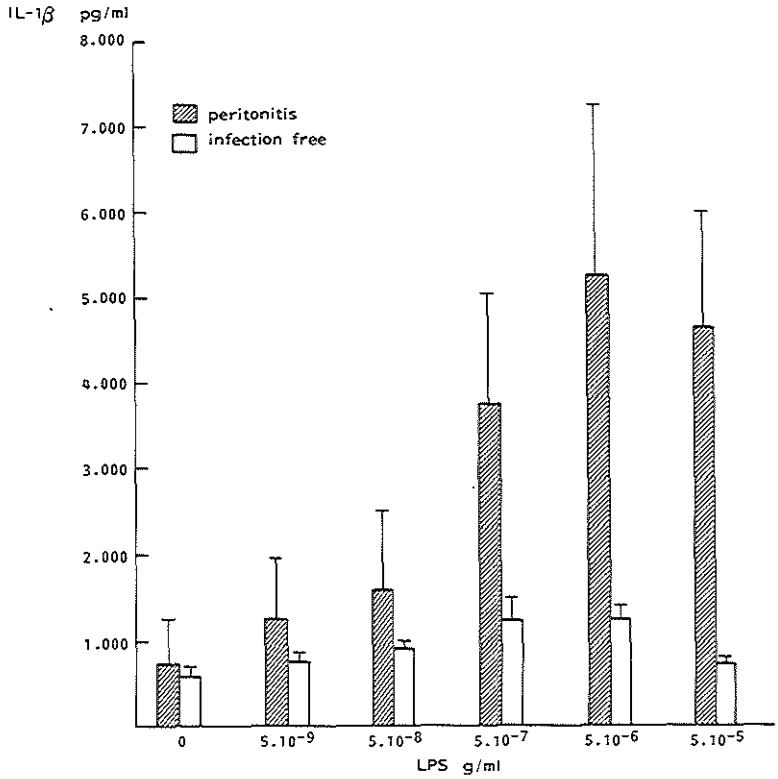

Fig. 2 IL-1ß release (means ± SEM) by peritoneal macrophages collected either during an infection-free period (n=3) (open bars) or during peritonitis (n=3) (hatched bars) in response to graded concentrations of LPS in the culture medium.

DISCUSSION

In the present study the relationship of the secretion of anti-inflammatory (PGE_2, PGI_2) and pro-inflammatory (IL-1ß) mediators by peritoneal macrophages from CAPD patients, either collected during an infection-free period or during an episode of peritonitis was determined. In order to compare the release of prostanoids and IL-1ß under the same conditions, in the present study prostanoid release was determined in the same 24 hours culture samples as used for measuring IL-1ß contents.

The present findings show that peritonitis induces profound changes in the secretion of the mediators in question. Peritonitis macrophages show a

sharp increase in IL-1ß release, provided that these cells are stimulated with LPS. The basal IL-1ß release, which may be affected by possible contaminations with the ubiquitous LPS, was similar for infection-free and peritonitis macrophages. These results confirm our previous findings which show that for IL-1ß release a stepwise activation of macrophages is required (3). Starting from this concept, it appears that during peritonitis macrophages are primed in the infectious inflammatory environment to become fully activated for IL-1ß release after in vitro stimulation ("triggering") with LPS. On the other hand, the basal release of PGI_2 and to a less degree of PGE_2 is decreased during peritonitis. These results, obtained with 24 hours measurement, are consistent with the previous findings when only a 2 hours period was covered (6,7).

Fierer e.a. studied prostanoid release by murine peritoneal macrophages after in vivo infection with Trypanosoma (12). While 6 days after infection, both basal and LPS stimulated PGE_2 secretion was increased, progression of the infection induced a decrease in PGE_2 release, which could not be enhanced by LPS late in infection. In contrast, PGI_2 release decreased progressively from the start of infection. Our results resemble the findings obtained 12 days after the Trypanosoma infection, in that the decrease of the basal PGE_2 secretion is reversed after stimulation with LPS. In our study, too, decrease of PGI_2 secretion was far more pronounced. The differences between the two prostanoids might be attributed to impaired prostacyclin synthetase activity (12). Failure of LPS to induce an increased PGI_2-release, as is found in our study concerning macrophages from both infected and uninfected patients, agrees with the findings of Schade, concerning mouse peritoneal macrophages (13), but contrasts to what is found in other studies (12, 14). Although equal pure macrophages populations were obtained after the purification procedure, a slight number of lymphocytes and PMN's were present in the cell preparations from infection-free and peritonitis patients respectively. It is found however, that lymphocytes are not capable of releasing prostaglandins, while PMN's release prostaglandins to a less extent than macrophages, particularly when incubated for longer time periods. (14). Therefore, it seems unlikely that the presence of the slight number of lymphocytes and PMN's in the cell cultures had a significant influence on the prostanoid contents as found in the present study.

Once set in motion, the inflammatory response is regulated especially by the secretory products of mononuclear phagocytes (15). The increased capability to secrete IL-1ß during peritonitis, as found in the present study,

enables the macrophages to amplify the inflammatory response.
Peritoneal macrophages of uninfected CAPD patients were found to be derived freshly frome bone marrow (16) and could be characterized as exudate macrophages, resident cells being very scant (17).
This does not preclude that peritonitis macrophages may be an even more immature population of cells, which might have contributed to their increased capacity for IL-1ß release, as found in this study.
IL-1ß production in cultured human monocytes is found to be regulated at multiple levels, including transscription and secretion (18). The biologically active 17 kD form of IL-1ß is present extracellulary. IL-1ß may also be present within the cell, almost entirely in its biologically inactive precursor form. (18). As we did not determine cell associated IL-1ß, it cannot be decided, whether total IL-1ß synthesis and secretion is increased during peritonitis and/or only the release of IL-1ß is increased.
The question remains to be answered, whether the distinct changes with regard to the secretion of prostanoids and IL-1ß as found in the present study, are merely coincidental or whether they are due to a causal relationship between the two events. Evidence for a role of PGE_2 and PGI_2 as endogenous regulators of IL-1ß production was found concering murine resident peritoneal macrophages and human monocytes as wel (9,19). With regard to PGI_2 it is tempting to speculate that its impaired release during peritonitis has enabled the macrophages to secrete more IL-1ß after stimulation with LPS. On the other hand, during peritonitis about the same amounts of PGE_2 are released by LPS stimulated macrophages as during an infection-freeperiod. These findings may indicate a distinct role for each one of the two prostanoids in the regulation of LPS stimulated IL-1ß secretion. Evidence that PGE_2 and PGI_2 differ in the regulatory function at inflammatory sites has been reported earlier. (20).

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Chapter 7

Tumor Necrosis Factor from peritoneal macrophages

(published paper)

Peritoneal Macrophages from patients on Continuous Ambulatory Peritoneal Dialysis have an increased capability to release Tumor Necrosis Factor during peritonitis

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SUMMARY

We have reported previously that human peritoneal macrophages collected from patients on Continuous Ambulatory Peritoneal Dialysis (CAPD) during an episode of peritonitis secrete increased amounts of interleukin-1 (IL-1), as compared to those collected during an infection-free period, provided the cells were stimulated *in vitro* by LPS. We now report that such macrophages release also higher amounts of Tumor Necrosis Factor (TNF), if collected during peritonitis and stimulated subsequently *in vitro* by LPS. The increase in release of TNF was ascertained by radio-immunoassays as well as by bioassay of cytostatic effect against the highly sensitive TNF target-cell line L929 murine transformed fibroblasts. The present reported results, in addition to previously reported data on release of IL-1, indicate that induction of release of cytokines from human peritoneal macrophages is a dual stepwise process: first priming *in vivo* in an inflammatory environment and, secondly stimulation *in vitro* by LPS.

INTRODUCTION

Peritoneal macrophages obtained from effluent dialysate of patients on Continuous Ambulatory Peritoneal Dialysis (CAPD), because of end stage renal failure, produce various mediators. We found previously that peritoneal macrophages of CAPD patients obtained during an episode of infectious peritonitis, a major complication of this treatment modality, show a decrease in the spontaneous *in vitro* release of prostanoids, as compared to an infection-free period. (1,2). On the other hand, the *in vitro* secretion of the pro-inflammatory cytokine interleukin-1ß (IL-1ß) is increased during peritonitis, provided that the macrophages are stimulated with LPS, indicating that activation for IL-1ß secretion by macrophages occurs stepwise. (3). Tumor necrosis Factor α (TNF-α), also referred as cachectin, is another pro- inflammatory cytokine. This 17-kD polypeptide shares many properties with IL-1. Like IL-1ß, TNF-α exerts its effects in autocrine, paracrine and endocrine fashion. Although it is produced by various cells, mononuclear phagocytes are its main source. (4,5). It is well accepted, that TNF plays a role in antibacterial resistance and experimental studies provide indeed evidence that TNF may enhance resistance to infection (6). Thus, it is conceivable that, under conditions of infectious peritonitis caused by microorganisms contamination (3), TNF release from

environmental macrophages might be increased. In the present study we investigated the *in vitro* release of TNF-α by peritoneal macrophages of CAPD patients, with the intention to make a comparison in this respect between peritoneal macrophages collected during an episode of infectious peritonitis and those collected during an infection-free period.

For this purpose, the *in vitro* release of TNF-α by peritoneal macrophages of CAPD patients, either in presence or absence of LPS, was determined in 12 episodes of peritonitis and 12 infection-free periods, using an immunoradiometric assay (IRMA), and in 12 episodes of peritonitis and 21 infection-free periods using a bioassay. TNF-α was determined concomitantly by immunoassay and bioassay in 9 episodes of peritonitis and 8 infection-free periods. The specificity of bioassay for TNF-α activity was ascertained by neutralisation tests with anti TNF-antibody.

MATERIALS AND METHODS

Study population

Macrophages were isolated from drained dialysate of CAPD patients using commercially available dextrose containing dialysis solutions (Dianeal^{R}, Baxter Laboratories, Deerfield, Illinois, USA). The peritoneal cells were harvested from one of the first bags with cloudy dialysate, prior to the administration of antibiotics. The diagnosis was confirmed by an elevated dialysate leukocyte count (>100/mm^3) consisting of over 50% polymorphonuclear cells (PMN's) and/or a positive microbial culture (7). A total number of 11 peritonitis patients were examined. From 4 out of 11 patients, cells were collected during 2 different episodes of peritonitis, thus bringing the total number of peritonitis episodes examined up to 15. In 9 peritonitis episodes supernatants were assayed for TNF-α using both IRMA and bioassay, in the other 6 episodes, either IRMA or bioassay was used. During 25 infection-free periods in 21 patients, cells were harvested from the morning bags. (From 4 out of 21 patients two samples were obtained). IRMA was used for TNF-α assay in supernatants from 12 infection-free periods and bioassay was used in 21 infection-free supernatants. Both IRMA and bioassay were used simultaneously in supernatants from 8 infection-free periods. Nine patients took part in the study both during an infection-free period and peritonitis. No patient was studied within 2

months of a peritonitis episode neither within 2 months after starting CAPD. After draining the dialysate was kept at 4°C until the cells were harvested within 24 hours. Informed consent was obtained from all the patients.

Macrophage preparation

The cells were collected from the dialysate by centrifugation (5 min. at 4°C, 700 g) and washed twice with Dulbecco's Modified Eagle's Medium (DMEM) (Gibco LTD., Paisly, Scotland). The macrophages were isolated by centrifugation (30 min at 4°C, 400 g) through a Percoll^{R} solution (Pharmacia LKB Biothechnology, Uppsala, Sweden) with a density of 1.064 g/ml. (8) Subsequently the cells were washed twice in DMEM and resuspended in RPMI 1640, supplemented with 10% fetal calf serum, 2 mM/L glutamin, 100 u/ml streptomycin-penicillin (Flow Laboratories, Irvine, Scotland) and 2g/L $NaHCO_3$ (Merck, Schuchhardt, FRG). The cells were counted in a Bürker chamber. Viability, as judged by trypan blue exclusion was found to exceed 95%. After centrifuging the cell suspension, the smears were stained with Hemacolor^{R} rapid blood smear staining (Merck, Darmstadt, FRG) and with non specific esterase staining (9). According to differential counting, the Percoll^{R} separated cell population was composed of 93 ± 7% macrophages, 5 ± 5% lymphocytes and 2 ± 2% PMN's when obtained during an infection-free period, and of 89 ± 5% macrophages, 1 ± 1 lymphocytes and 10 ± 5% PMN's when obtained during peritonitis (values expressed as mean ± SD). One ml of 1.0 x 10^6 cells/ml suspension was placed in 35 mm diameter tissue culture wells (Costar, Cambridge, MA, USA) and cultured at 37°C for 24 hours in a humidified atmosphere containing 7.5% CO_2, in absence or presence of 5 µg/ml of LPS (E. coli 0111:B4) (Sigma chemical company, St. Louis, MO, USA). After the culture period, the supernatants were filter sterilised over a 0.22 µm Millex filter (Millipore SA, Molsheim, France) and stored at -70°C.

Immunoradiometric assay. (IRMA)

Concentrations of TNF-α in supernatants were determined using a commercially available two site immunoradiometric assay (IRMA),

according to manufacturers instructions (Medgenix, Brussels, Belgium). Briefly, monoclonal antibodies, directed against distinct epitopes of TNF-α, are attached to the lower and inner surface of plastic tubes. After incubating the standards or samples in the coated tubes in the presence of I^{125}-labelled antibodies to TNF-α for 20 hours, TNF-α is bound to both the labelled and coated antibodies. After washing, the radioactivity bound to the tube, reflects TNF-α concentrations. The assay is sensitive to 15 pg/ml. In each separate IRMA-test, samples from both infected and uninfected patients were determined in duplicate.

Bioassay

TNF-activity in the supernatants was determined by measuring the cytotoxic/cytostatic effects of TNF-α on the murine fibroblast-like cell line L 929 (a gift from Dr. W. Fiers, State University of Ghent, Belgium) (10,11). 25 µl of a suspension of 40 x 10^6 L 929 cells/ml and 50 µl of RPMI 1640 culture medium was pipetted in each well of a 96-well flat bottom microtiterplate (NUNC, Roskilde, Denmark). To quadruplicate wells, 25 µl of medium (control), human recombinant TNF standard solution (Roche Research, Ghent, Belgium) or macrophage supernatant was added.
After incubation for 24 hours in humidified atmosphere (7.5% CO_2) at 37° C, 50 µl of a 10 µCi ^3H thymidine/ml solution was added (Amersham Laboratories, Amersham, England). After 18 hours of incubation, L 929 cells were harvested on glassfiber filtermats (Skatron Inc., Sterling, VA, USA) whereupon ^3H-thymidine uptake was measured by liquid scintillation spectroscopy. The percentage of growth inhibition (cytostasis) was calculated according to the formula.

$$\frac{\text{(control cpm-experimental cpm)} \times 100\ \%}{\text{control cpm}} \quad (12)$$

To determine the specificity of the assay, 3 supernatants obtained after incubation of peritoneal macrophages from 3 infected CAPD patients in the presence of 5µg/ml of LPS, were preincubated for 20 hours in tubes coated with monoclonal antibodies to TNF-α (Medgenix).

To examine, whether IL-1 has an additive or synergistic cytostatic effect on L 929 cells, human IL-1 and TNF-α were added at various concentrations to L 929 cell suspension. This in view of the fact that incubation of macrophages with LPS induces also release of IL-1(3).

Statistics

Data were expresses as mean ± standard error of the mean. Statistical significane was determined by Wilcoxon test.

RESULTS

Characterization of patients

The characteristics of patients on CAPD included in the study, is given in table 1.

Table 1. Characteristics of CAPD patients participating in the study.

Patient no.	sex	age (years)	primary renal disease
1.	F	54	diabetic nephropathy
2.	F	54	hemolytic uremic syndrome
3.	M	76	hypertensive nephropathy
4.	M	59	diabetic nephropathy
5.	F	32	chronic glomerulonephritis
6.	F	21	diabetic nephropathy
7.	F	60	chronic pyelonephritits
8.	M	52	focal glomerulosclerosis
9.	M	65	unknown
10.	F	27	fibromuscular dysplasia
11.	F	58	unknown
12.	M	36	focal glomerulosclerosis
13.	M	70	hypertensive nephropathy
14.	M	34	chronic glomerulonephritis
15.	M	71	cholesterol emboli
16.	F	40	chronic glomerulonephritis
17.	M	40	diabetic nephropathy
18.	M	73	hypertensive nephropathy
19.	M	72	hypertensive nephropathy
20.	M	65	diabetic nephropathy
21.	F	48	diabetic nephropathy
22.	F	48	diabetic nephropathy
23.	F	51	polycystic disease

A total number of 23 patients were included consisting of 11 females and 12 males. The primary renal disease was diagnozed as diabetic nephropathy, hemolytic uremic syndrome, hypertensive nephropathy, chronic glomerulonephritis, chronic pyelonephritis, focal glomerulosclerosis, fibromuscular displasia, cholesterol emboli and polycystic disease. In 11 CAPD patients with peritonitis, dialysate WBC were counted and the causative microorganism was determined (table 2).

Table 2. Dialysate leukocyte counts and assays used for determination of TNF-α contents in supernatants obtained during 15 episodes of peritonitis in 11 CAPD patients.

patient no.	WBC[1] (10^6/L)	causative microorganisms	TNF-α (IRMA)	TNF-α (L929)
2.	1163	candida albicans	+	+
	5310	staph. aureus	+	-[2]
3.	2220	strept. viridans	+	+
	3600	staph. epidermidis	-	+[3]
4.	160	acinetobacter	+	+
	1795	staph. epidermidis	-	+
5.	2416	staph. epidermidis	+	+
6.	2092	acinetobacter	+	+
9.	9576	staph. epidermidis enterococ	+	+
14.	1418	staph. aureus	+	-
	296	strept. viridans	-	+
17.	1140	pseudomonas	+	+
18.	2592	enterococcus	+	+
22.	3040	strept. viridans	+	+
23.	184	staph. aureus	+	-

1). dialysate white blood cell count.
2). solely IRMA carried out.
3). solely bioassay carried out.

TNF-α determinations by IRMA and/or bioassay method were performed with supernatants collected from 15 episodes of peritonitis. In 9 out of 12 cases, the TNF-α determinations were done in parallel by both methods. WBC counts and TNF determinations were also done with cells collected from 21 CAPD patients during 25 infection-free periods (table 3). The TNF-α determination by IRMA were done in 12 cases and by bioassay in

21 cases. In 8 infection-free periods, TNF determinations by both IRMA and bioassay were performed concomitantly.

Table 3. Dialysate leukocyte counts and assays used for determination of TNF-α contents in supernatants obtained during 25 infection-free periods in 21 CAPD patients.

patient no.	WBC[1] (10^6/L)	TNF-α (IRMA)	TNF α (L929)
1.	9	+	-[2]
	10	-	+[3]
2.	10	+	+
	4	-	+
3.	2	+	-
	1	-	+
4.	20	+	+
	13	+	+
5.	22	+	-
6.	17	+	+
7.	6	+	+
8.	7	+	+
9.	3	+	+
10.	3	+	+
11.	2	+	-
12.	2	-	+
13.	4	-	+
14.	5	-	+
15.	8	-	+
16.	4	-	+
17.	32	-	+
18.	1	-	+
19.	2	-	+
20.	28	-	+
21.	6	-	+

1). dialysate white blood cell count.
2). solely IRMA carried out.
3). solely bioassay carried out.

Radioimmunoassay

In the absence of LPS, peritoneal macrophages secreted detectable amounts of TNF-α in 10 out of 12 infection-free periods (mean 1,070 ± 277 pg/ml). Below detection a value of 15 pg/ml was assigned. In the presence of 5

µg/ml of LPS, significantly more TNF-α was released (mean 2,601 ± 377 pg/ml, p< 0.005).
When macrophages were collected during peritonitis, a detectable release of TNF-α was found in all 12 peritonitis episodes (mean 1,031 ± 277 pg/ml), in the absence of LPS in the culture medium. With 5µg/ml of LPS, *in vitro* release of TNF-α from macrophages collected during peritonitis was significantly increased to values of 9,360 ± 4,256 pg/ml (p < 0.005). When infection-free and peritonitis-macrophages are compared, no difference in TNF-α release is found, in the absence of exogenous LPS. However, LPS stimulated macrophages displayed significantly higher TNF-α release during peritonitis in comparison with the infection-free period (p < 0.05). Individual determinations are presented in fig. 1A and mean values are brought in fig 1B.

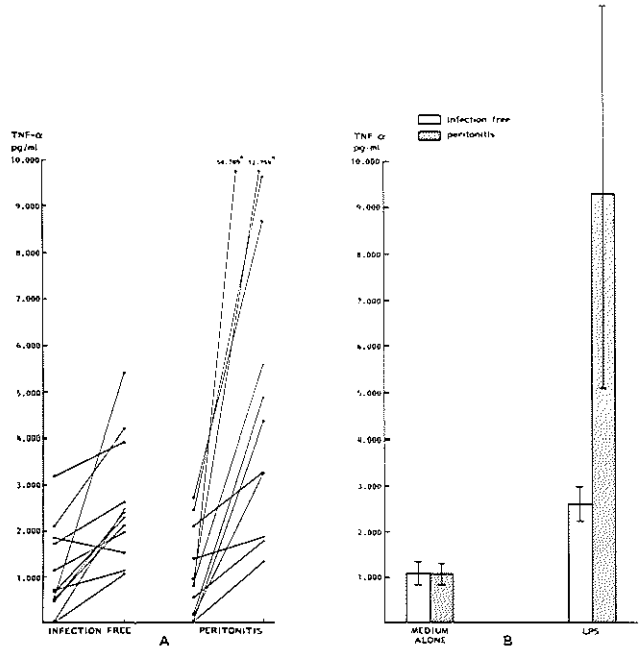

Figure 1. Release of TNF-α in vitro, as determined by immunoradiometric assay, by peritoneal macrophages of CAPD patients.
A. a line connects the data obtained for each *individual observation* in the absence (●) or presence (○) of 5 µg of LPS/ml during an infection-free period (left) and an episode of peritonitis (right). * pg/ml.
B. Comparison of TNF-α release in vitro *(means ± SEM)* either in the absence (left) or presence (right) of 5 µg of LPS/ml during an infection-free period (open bars) and peritonitis (hatched bars).

Figure 2 shows mean TNF-α release by peritoneal macrophages during 3 infection-free periods and 3 episodes of peritonitis, in response to graded doses of LPS. Both in the infection-free and the peritonitis macrophages, maximum TNF-α release was attained by stimulation of LPS at a dose of 5 µg/ml. ($5x10^{-6}$g/ml). Peritonitis macrophages showed a definite LPS dose dependent increase within the range of $5x10^{-6}$ to 5 x 10^{-9} g/ml.

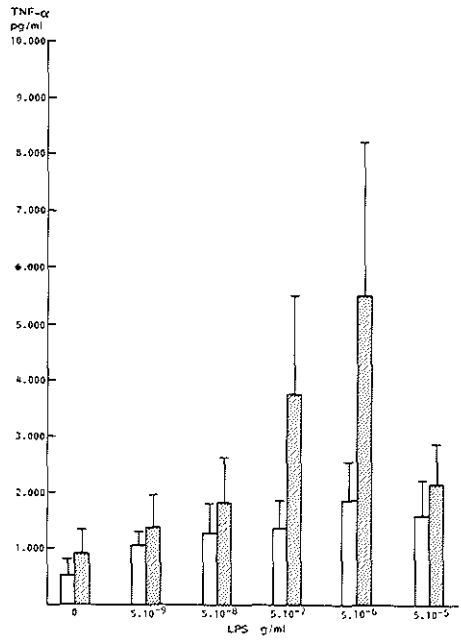

Fig. 2 Release of TNF-α in vitro (means ± SEM), as determinded by immunoradiometric assay, by peritoneal macrophages of CAPD patients, in response to graded concentrations of LPS in culture medium. The cells were collected either during an infection-free period (n=3) (open bars) or during peritonitis (n=3) (hatched bars).

Bioassay

The secretion of bioactive TNF-α, as determined by its cytotoxic/cytostatic effects on the fibroblast-like cell line L 929, is shown in figure 3. For infection-free peritoneal macrophages, percentage inhibition of ^{3}H thymidine uptake, a measure for cytostasis, increased from 4.33% ± 5.14, when the macrophages were cultured in the absence of LPS, to 15.76% ± 6.52 when these cells were incubated with LPS ($p < 0.0681$).

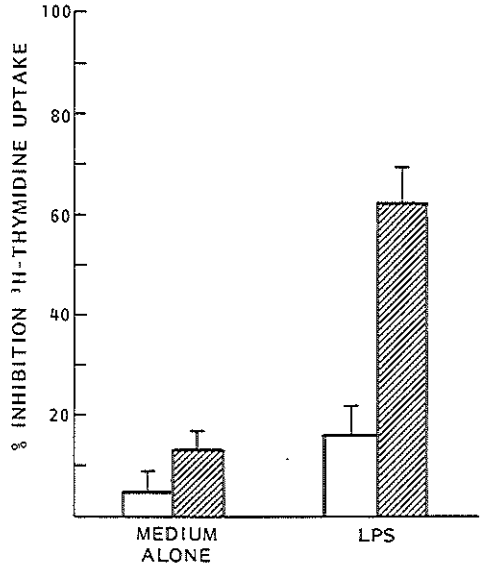

Fig. 3 Release of bioactive TNF-α in vitro, as determined by % inhibition of ^{3}H-thymidine uptake by L 929 target cells (means ± SEM), by peritoneal macrophages of CAPD patients. The cells were collected during an infection-free period (open bars) or during peritonitis (hatched bars) and cultured in medium alone (left) or in the presence of 5µg LPS/ml.

For peritonitis macrophages, percentage inhibition increased significantly from 12.92 ± 4.09, in the absence of LPS to 61.92% ± 7.72 when cultured in the presence of LPS ($p < 0.005$). When infection-free and peritonitis macrophages are compared a significant higher TNF-α activity in the supernatants was found when the peritoneal macrophages, obtained during peritonitis, were incubated in the presence of LPS ($p < 0.005$). In the absence of LPS, the difference between infection-free and peritonitis macrophages did not reach statistical significance ($p < 0.0643$). Figure 4 shows TNF-α activity in supernatants when peritoneal macrophages were obtained during 4 infection-free periods and 4 episodes of peritonitis in response to graded doses of LPS. Both with regard to the infection-free and peritonitis macrophages, maximum TNF-α activity was attained, when the cells were stimulated by LPS, at a dose of 5 µg/ml.

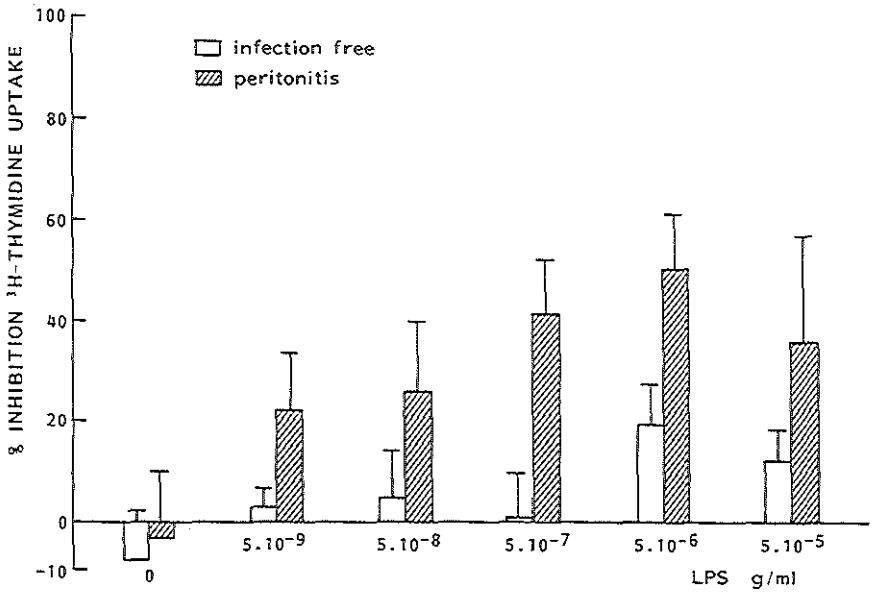

Fig. 4 Release of bioactive TNF-α in vitro, as determined by % inhibition of ^{3}H-thymidine uptake by L 929 target cells (means ± SEM) by peritoneal macrophages in response to graded concentrations of LPS in culture medium. The cells were collected either during an infection-free period (n=4) (open bars) or during peritonitis (n=4) (hatched bars).

Peritonitis macrophages displayed, also by this method, a definite LPS dose dependant increase. Within the range of $5x10^{-6}$ - $5x10^{-9}$ g/ml, preincubation of three supernatants in anti-TNF-α coated tubes, resulted in a sharp drop of the growth inhibition from 68.0 % ± 8.7 to 15.3 % ± 6.8 (mean ± SEM), indicating that cytostatic activity was mediated by TNF-α. In view of the fact that most likely macrophages secrete also IL-1 in addition to TNF-α, the effect of adding IL-1 to known amounts of TNF-α was determined. Table 4 shows that addition of IL-1 did not augment TNF-α induced cytostasis.

Table 4. Effect of IL-1 addition on % growth inhibition of L 929 target cells by TNF.

compound added	% Inhibition.
None	*
TNF 1U/ml[1]	24
TNF 10U/ml	65
TNF 1U/ml + IL-1 10U/ml[2]	23
TNF 10U/ml + IL-1 10U/ml	67
TNF 10U/ml + IL-1 100U/ml	70
IL-1 10U/ml	19
IL-1 100U/ml	-4

* mean cpm: 22,751 ± 2,496 (mean ± SD) in control.
1. specific activity: 7×10^7 U/mg human recombinant TNF-α.
2. specific activity: 1×10^8 U/mg human recombinant Il-1α.

DISCUSSION

The aim of the present study was to determine to what extent an inflammatory environment affects the in vitro release of TNF-α by peritoneal macrophages obtained from CAPD patients. The present findings show that secretion of immunoreactive TNF-α is increased upon stimulation with LPS both during an infection-free period and during an episode of peritonitis. When TNF-α release by infection-free and peritonitis macrophages are compared, LPS stimulated peritoneal macrophages obtained during peritonitis released considerably higher amounts of TNF-α in comparison with infection-free macrophages. However, the same amounts of TNF-α were released whether the macrophages were obtained during peritonitis or during an infection-free period. Obviously, peritonitis macrophages show an increase in TNF-α release provided that these cells are additionally stimulated with LPS. These findings are similar to those obtained on IL-1ß determinations, as recently reported by our group. (3). This indicates that peritoneal macrophages of CAPD patients, which are blood monocyte derived cells (13), are also stepwise activated for TNF-release. Starting from this concept, it appears that during peritonitis macrophages are primed in the infectious inflammatory environment to become fully activated for TNF-α release after "triggering" with LPS.

In addition to the determination of TNF-α by radioimmunoassay, we determined also TNF activity against the selectively sensitive cell line L 929. In this context it should be noted that macrophages in addition to TNF, might release another cytostatic factor, especially when stimulated with LPS (14). Increase in release of IL-1 by macrophages from peritonitis episodes should also be considered. Our tests showing neutralisation of L 929 cytostasis by anti TNF antibody prove however, that indeed cytostasis is due to presence of TNF in the supernatants. Our findings and those reported by others (15) demonstrate that IL-1 does not induce cytostasis or cytotoxicity on L 929 cell line nor synergizes with human recombinant TNF-α in these bioassays. Therefore, it is unlikely that presence of IL-1 in the supernatants has affected TNF induced cytostasis. The findings obtained are in line with those obtained for immunoreactive TNF-α. This applies especially to the difference in TNF-α contents between the supernatants from infected and those from uninfected patients, when the macrophages were stimulated with LPS. This means, that peritonitis macrophages have an increased capability to secrete bioactive TNF-α. In studies on bioactive TNF-α secretion by human monocytes, it was shown that > 95% of total (supernatant + cell lysate) TNF activity after stimulation with LPS, was found in the supernatant of fresh and cultured cells. (16) In contrast, about 60% of total IL-1 activity of fresh monocytes, but only 10% of total IL-1 activity of cultured cells was secreted. Hazuda e.a. found, concerning human blood monocytes, that TNF-α, in contrast to IL-1, was found exclusively outside the cells. (17). In our previous study on IL-1, it could not be decided, whether the increased IL-1ß secretion by peritoneal macrophages, as found during peritonitis after stimulation with LPS, could be attributed to increased synthesis or exclusively to increased secretion of IL-1ß produced (5). Although in the present study too, TNF-α was determined only in supernatants, it seems plausible, in the light of the studies aforementioned, that the increased release of TNF-α as found during peritonitis after stimulation with LPS, results from an increased synthesis of TNF-α.
This corrobates the concept, that during peritonitis macrophages are primed for enhanced TNF-α synthesis, which, at the molecular level, may be based on increased transscription.

The present study shows, that during peritonitis, in addition to the migration of large numbers of mononuclear phagocytes to the peritoneal cavity, the capability to release the proinflammatory cytokine TNF-α is

increased on a per cell basis. This may contribute to enhanced antibacterial resistance. (6).

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Chapter 8

Regulation of Interleukin-1 and Tumor Necrosis Factor release from peritoneal macrophages by prostaglandin E_2

(published paper)

Prostaglandin E_2 inhibits the release of tumor necrosis factor-α, rather than interleukin-1ß, from human macrophages.

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SUMMARY

We reported previously that macrophages obtained from renal patients on Continuous Ambulatory Peritoneal Dialysis (CAPD) during an episode of infectious peritonitis, display a decrease in intracellular cAMP levels and in spontaneous *in vitro* release of PGE_2 and PGI_2 . Such macrophages release also large quantities of IL-1ß and TNF-α when stimulated *in vitro* by LPS. In view of the interregulatory effects between PGE_2 and macrophage cytokines (IL-1ß and TNFα) in their production, we examined in the present work to what extent the LPS-induced release of either IL-1ß or TNFα *in vitro* from CAPD-originated peritoneal macrophages, is affected by graded doses of exogenous PGE_2 (range 0-1000 ng/ml) or by the cyclooxygenase inhibitor indomethacin (INDO) (10^{-6} M). IL-1ß and TNFα were determined using an enzyme linked immunoabsorbent assay and an immunoradiometric assay, respectively. We found that PGE_2 induced invariably a dose dependent decrease of TNFα release: In peritoneal macrophages collected during an infection-free period, TNFα release decreased from 3225 pg/ml (controls) tot 353 pg/ml at 1000 ng/ml pf PGE_2 and in peritoneal macrophages collected during an episode of infectious peritonitis from 4100 pg/ml (controls) to 545 pg/ml at 1000 ng/ml of PGE_2. However, PGE_2 failed to influence the secretion of IL-1ß. INDO induced an approx. two fold increase in TNFα release, but had no effect on IL-1ß release. These findings indicate, that exogenous and endogenous PGE_2 controls TNFα rather than IL-1ß release from LPS stimulated peritoneal macrophages.

Key words: Tumor Necrosis Factor α; Interleukin-1ß; Prostaglandin E_2; LPS, bacterial lipopolysaccharide; Indomethacin; Macrophages, peritoneal human.

Introduction

In previous studies we examined the intracellular levels of the second messenger cyclic AMP(cAMP), the *in vitro* release of the prostanoids PGE_2 and PGI_2 (1,2,3) and of the cytokines interleukin-1ß (IL-1ß) (4) and tumor necrosis factor (TNFα) (5) from human peritoneal macrophages obtained from renal patients on Continuous Ambulatory Peritoneal Dialysis (CAPD). Macrophages obtained during an episode of infectious peritonitis

displayed a decrease in the intracellular cAMP levels and in the spontaneous *in vitro* release of PGE_2 and PGI_2, as compared to macrophages collected during an infection-free period (1,3). In contrast, the *in vitro* release of IL-1ß (4) and of TNFα (5) from macrophages collected during peritonitis was markedly increased following stimulation by LPS. We postulated that these distinct changes with regard to the secretion of cytokines and prostanoids may be interrelated. Evidence for a role of prostaglandins as endogenous regulators of IL-1 production by murine resident peritoneal macrophages and human monocytes (6,7) has been reported. PGE_2 was also found to have a suppressive effect on the production of TNFα by mouse (8) or rat (9) peritoneal macrophages. Although TNFα and IL-1 share a wide spectrum of biological activities (10), some dissimilarities in their regulation of production have been reported: a) dissimilarity in kinetics of production by human peripheral blood mononuclear cells (11); b) differential production by human monocytes pretreated with IFN-γ before LPS stimulation (12); c) difference in regulation of production by human alveolar macrophages (13) and by murine peritoneal macrophages (14), stimulated with LPS; and d) distinct patterns of IL-1 and TNFα production by cells pretreated with phorbol-1-myristate acetate before stimulation with LPS (15). In the present study we aimed to examine the role of prostaglandins in the regulation of IL-1ß and TNFα release from human peritoneal macrophages. Human peritoneal macrophages collected from both infected and uninfected renal patients on Continuous Ambulatory Peritoneal Dialysis, were cultured in the presence of graded doses of exogenous PGE_2 or a standard dose of the cyclooxygenase inhibitor indomethacin (INDO).

MATERIALS AND METHODS

Clinical data

A total number of 19 patients (9 men and 10 women) participated in this study. The patients were treated for at least 2 months with Continuous Ambulatory Peritoneal Dialysis (CAPD) because of end stage renal failure (16). Commercially available peritoneal dialysis solutions consisting of 132 mmol/l Na^+, 1.75 mmol/l Ca^{2+}, 0.75 mmol/l. Mg^{2+}, 102 mmol/l Cl^-, 35 mmol/l lactate and 1.36%, 2.27% or 3.86% anhydrous dextrose (Dianeal ^{R}, Baxter Laboratories Deerfield, IL, USA) were changed four times a day.

Macrophages were isolated from effluent dialysate during an episode of infectious peritonitis or during an infection-free interval. From 13 patients who developed 20 episodes of peritonitis, cells were harvested from one of the first bags in which opalescence, due to increased leukocyte numbers, appeared, i.e. prior to the intraperitoneal administration of antibiotics. The diagnosis was confirmed by an elevated leukocyte count (>100/mm^3) in the effluent dialysate with over 50% polymorphonuclear cells (PMN's) at differential counting and/or a positive microbial culture (16). The causative microorganisms in 20 episodes of peritonitis that developed in 13 patients were identified as *Staphylococcus epidermidis* (7x), *Staphylococcus aureus* (3x), *Streptococcus viridans* (3x) *Acinetobacter* (2x), *Pseudomonas* (2x), *Enterococcus* and *Candida albicans*. In one episode of peritonitis no microorganisms could be identified. Patients with a relapsed peritonitis, i.e. recurrence of peritonitis within 3 weeks, and patients with peritoneal eosinophilia were excluded from the study. All these 13 patients were examined during an episode of peritonitis and eight out of the 13 were also examined during an infection-free period. The other six patients were examined during an prolonged infection-free period. In total, 20 samples were examined from 13 patients with peritonitis and 20 samples from 14 patients during an infection-free period. No patient was studied within 2 months after starting CAPD.

Collection and culture of macrophages

After drainage of the peritoneal dialysate, the bags were kept at 4°C where upon the cells were harvested within 24 hours. The cells were collected from the dialysate by centrifugation (5 min. at 4°C, 700 g), washed twice with DMEM (Gibco LTD., Paisly, Scotland) and separated through a Percoll^R solution (Pharmacia LKB Biotechnology, Uppsala, Sweden) with a density of 1.064 g/ml (17). Subsequently the cells were washed twice in DMEM, resuspended in RPMI 1640, supplemented with 10% fetal calf serum, 2mM/L glutamin, 100 U/ml streptomycin-penicillin (Flow Laboratories, Irvine, Scotland), 2g/L $NaHCO_3$ (Merck, Schuchhardt, FRG), and counted in a Bürker chamber. Viability, as judged by trypan blue exclusion was found to exceed 95%. After centrifuging the cell suspension, the smears were stained with Hemacolor^R rapid blood smear staining (Merck, Darmstadt, FRG) and with non-specific esterase staining (18). According to differential counting, the Percoll^R separated cell population

was composed of 93 ± 7% macrophages , 5 ± 5% lymphocytes and 2 ± 2% PMN's when obtained during an infection-free period, and of 89 ± 5% macrophages, 1 ± 1 lymphocytes and 10 ± 5% PMN's when obtained during peritonitis (values expressed as mean ± SD). One ml of 1.0 x 10^6 cells/ml suspension was placed in 35 mm diameter tissue culture wells (Costar, Cambridge, MA, USA) and cultured at 37°C for 24 hours in a humidified atmosphere containing 7.5% CO_2, with 5µg/ml of LPS in the presence of graded doses of PGE_2 (0, 10, 100, 1000 ng/ml) (Sigma, St. Louis, MO, USA) or with 10^{-6} M INDO, both in the presence or absence of 5µg/ml of LPS (E. coli 0111: B4 Sigma, St. Louis, MO, USA). We found that this concentration of INDO had no effect on the rate of ^{3}H-thymidine incorperation in the highly TNFα sensitive strain L 929. Moreover, INDO did not affect the antitumor cytostatic activity of TNFα against the same strain. (results not shown here). INDO was purchased from the Pharmacy Department, University Hospital "Dijkzigt", Rotterdam, The Netherlands. After the culture period, the supernatants were removed, filter sterilized over 0.22µm Millex filter (Millipore SA, Molsheim, France) and stored at -70°C.

TNFα assay

Concentrations of TNF-α in macrophage supernatants were determined using a commercially available two-site immunoradiometric assay (IRMA), according to manufacturers instructions (Medgenix, Brussels, Belgium). The assay is sensitive to 15 pg/ml. In each separate IRMA-kit, samples from both infected and uninfected patients were determined in duplicate.

IL-1ß assay

IL-1ß concentrations of peritoneal macrophage supernatants were determined using a commercially available enzyme linked immuno-absorbent assay (ELISA) (Cistron Biotechnology, Pine Brook, NJ, USA), as described elsewhere (4). IL-1ß concentrations were obtained from a standard curve. The assay is sensitive to 20 pg/ml. In each separate ELISA-kit, samples from both infected and uninfected patients were determined, in duplicate.

PGE_2 assay

For PGE_2 determinations, the macrophage supernatants were applied to Sep Pak silica cartridges (Waters Associates, Etten-Leur, The Netherlands) and eluted with ethanol. The extracts were evaporated to dryness and dissolved in RIA buffer. Prostaglandin Standards were obtained from the Sigma Chemical Company (St. Louis, USA). Prostaglandin E2 (Amersham, UK) and antibody (Advanced Magnetics, Cambridge, USA) were added. After incubation, charcoal suspension was added. After 15 minutes at 4°C, the tubes were centrifugated and decanted. Counts were measured in a beta-counter (Packard). The method was described in detail elsewhere (1). Data were expressed as means ± standard error of the means.

Statistical analysis

Statistical significance was determined using the Wilcoxon matched pairs signed rank test. Two sided P values < 0.05 were considered to be statistically significant.

RESULTS

Peritoneal macrophages were cultured with 5µg/ml of LPS in the presence of graded doses of PGE_2. Supernatants from 9 peritonitis episodes and 8 infection- free periods were determined for their TNFα concentrations. IL-1ß concentrations were measured in supernatants from 11 peritonitis episodes and 8 infection-free periods. As shown in figure 1, PGE_2 induced a sharp decrease in TNFα release in a clearly dose-dependent manner, that was found invariably in each individual experiment. This applies to macrophages from both infected and uninfected patients. Incubation with the highest doses of PGE_2 (1000 ng/ml) reduced TNFα release to roughly 10% of the control values. In contrast, PGE_2 had no appreciable effect on IL-1ß release.

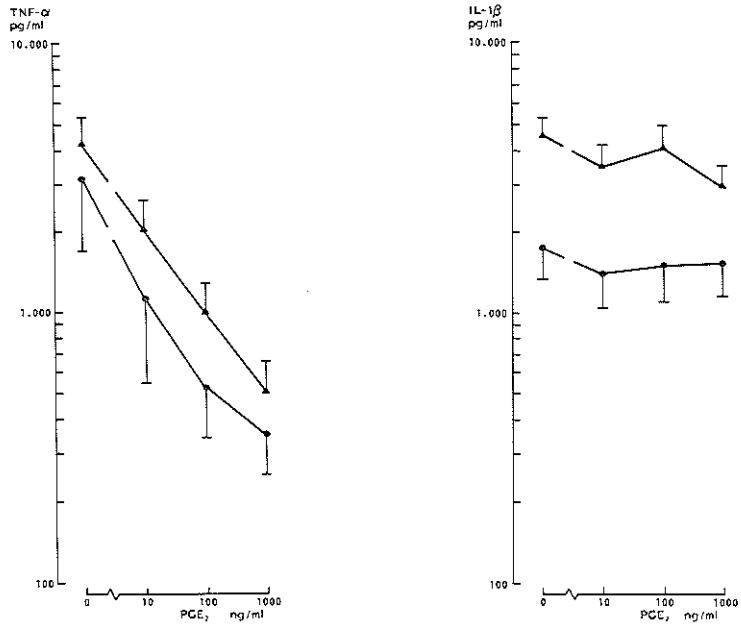

Fig. 1 Peritoneal cells were obtained from effluent dialysate of patients on CAPD, either during an episode of infectious peritonitis (▲) or during an infection-free period (●).
Supernatants from macrophages cultured with 5µg/ml of LPS in the presence of graded doses of PGE_2 from 9 peritonitis episodes and 8 infection-free periods were tested for their concentrations of TNFα using IRMA (left). Macrophage Supernatants from 11 peritonitis episodes and 8 infection-free periods were tested for IL-1ß contents (right).

In order to examine, whether endogenous prostaglandins have an influence on TNFα and IL-1ß release, peritoneal macrophages were cultured with 10^{-6} M of the cyclooxygenase inhibitor indomethacin (INDO), in the absence or presence of 5µg/ml of LPS. As expected, INDO induced a sharp decline in PGE_2 release (Table 1). In agreement with previous results (1), spontaneous PGE_2 release from peritonitis macrophages was markedly reduced. Macrophage supernatants from 14 episodes of infectious peritonitis and from 12 infection-free periods were determined for their TNFα concentrations.

Table 1. Effect of indomethacin on release of PGE_2 from human peritoneal macrophages.

Macrophage treatment [a]	PGE_2 (pg/ml).	
	infection-free macrophages (n=4)	Peritonitis macrophages (n=5)
None	16715 ± 8768[b]	9352 ± 8178
Indomethacin (10^{-6}M)	245 ± 208	213 ± 139
LPS (5µg/ml)	38193 ± 14402	34329 ± 17285
LPS + Indomethacin	191 ± 111	214 ± 97

[a] Peritoneal macrophages ($1x10^6$/ml) from 4 infection-free episodes and from 5 episodes of peritonitis were cultured for 24 hours. Concentrations of PGE_2 were determined in the macrophages supernatants by RIA.

[b] pg/ml, mean ± SD

As shown in figure 2A, culturing peritoneal macrophages from infection-free patients with INDO, induced a significant, approximately two fold increase in TNFα release, when the cells were stimulated with LPS. In the presence of LPS, peritoneal macrophages from infected patients too, displayed a significant increase in TNFα release ($p < 0.02$). (fig. 2A). IL-1ß release was measured in supernatants from 11 peritonitis episodes and from 10 infection-free periods. In contrast to the findings on TNFα, INDO had no effect on release of IL-1ß from infection-free and peritonitis macropahges, cultured in the presence or absence of LPS (Fig. 2B).

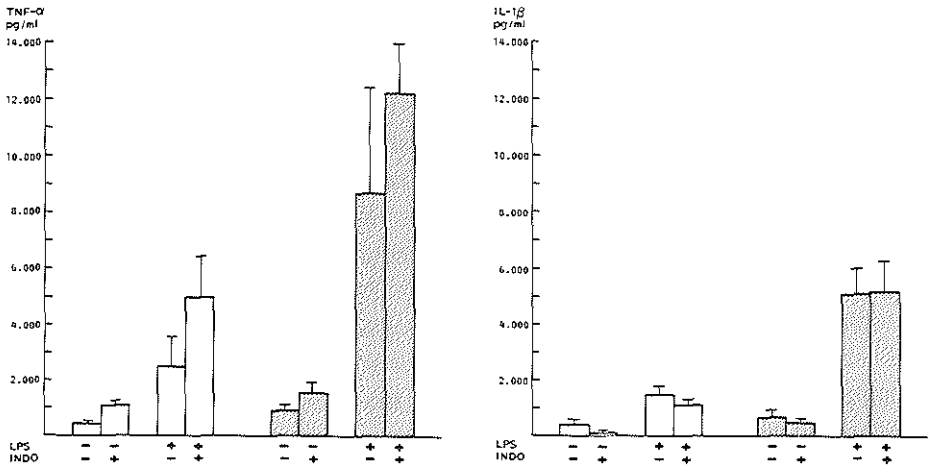

Fig. 2 Human peritoneal macrophages were collected either during an episode of infectious peritonitis (hatched bars) or during an infection-free period (open bars) 1.0 x 10^6 cells (90% macrophages) were cultured for 24 hours with (LPS+) or without (LPS-) 5µg/ml of LPS, either in the presence (INDO+) or absence (INDO-) of 10^{-6} M of indomethacin. Supernatants were tested for their concentrations of TNFα using IRMA and/or of IL-1ß using ELISA. Indomethacin induced a statistically significant increase in TNFα release from both LPS stimulated and unstimulated macrophages. In contrast, indomethacin did not affect IL-1ß release.

DISCUSSION

Prostaglandins are thought to have down-regulatory effects on various functions of macrophages, including the release of TNFα and IL-1ß, by elevation of the intracellular cAMP levels via activation of the adenylate cyclase complex. (8,19,20). It should be also noted that low doses of PGE_2 were found to stimulate release of TNFα from rat resident peritoneal macrophages (9). Our findings indicate that PGE_2 affects release of TNFα and IL-1ß in a quite distinct fashion. PGE_2 induced invariably a sharp, clearly dose dependent, decline in LPS stimulated TNFα release. In previous studies we found that similar doses of PGE_2 induced a cAMP rise (1,2,3), indicating that PGE_2 may suppress TNFα release via the second messenger cAMP. With regard to murine peritoneal macrophages it was found by others, that TNFα release and cell associated TNFα, as

determined by L929 fibroblast lytic bioassay and TNFα mRNA accumulation were suppressed under the influence of PGE_2 via a rise in cAMP levels (8,20,21,22). Our data on immunoreactive TNFα release from human peritoneal macrophages are in line with these findings. The INDO-induced increase in TNFα release, as found in the present study, provides evidence that also endogenous prostaglandins suppress TNFα release. In sharp contrast, PGE_2 induced no appreciable change in IL-1ß release. It is conceivable, that PGE_2 inhibits IL-1ß secretion via a cAMP independent pathway (23). It was found by others (6,7,24) that prostaglandins inhibit release of bioactive IL-1 from mononuclear phagocytes, whereas neither IL-1 mRNA accumulation nor cell associated IL-1 bioactivity was suppressed (21). This may indicate that solely secreted IL-1 bioactivity is inhibited by prostaglandins. We determined in the present work the IL-1ß component of IL-1. It is known that the biologically active 17 Kda form of IL-1ß is found as a secretory product outside the cell, whereas the biologically inactive 31 Kda precursor form is found both intra- and extracellularly (25,26). We cannot exclude the possibility that the ELISA test used by us, detects also precursor IL-1ß. Accordingly, we cannot rule out the possibility that prostaglandins affect the processing from precursor IL-1ß to mature IL-1ß. It was also found, that PGE_2 has no effect on IL-1 synthesis by macrophages, but rather has a direct effect on the proliferation of thymocytes, (27,28) We found now that INDO has no effect on IL-1ß release. Except being taken on the order of magnitude, the effect of PGE_2 and INDO on LPS-induced release of IL-1ß and TNFα, was similar for macrophages collected during an inflammation free period or during peritonitis.

The release of TNFα and IL-1ß from LPS stimulated macrophages of CAPD patients was also proven in our previous work (29) by a bioassay based on determination of cytostatic activity of macrophages supernatants against the TNFα sensitive strain L 929 (3,29) and against the IL-1ß sensitive strain, WEHI-3B. The antitumor cytostatic activity was specifically neutralized by either antibodies against TNFα (3,29) or against IL-1ß respectively (29).

TNFα and IL-1ß can exert many of their biological functions after being secreted by the cells. Our study provides evidence that release of TNFα rather than IL-1ß is controled by PGE_2. This distinct regulatory role of PGE_2 is in line with findings of others concerning the regulation of monokine gene expression. (21,22).

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Chapter 9

Summary and general discussion

9.1. Summary

This thesis deals with the role of several mediators released from peritoneal macrophages of patients which are on Continuous Ambulatory Peritoneal Dialysis (CAPD) because of end stage renal failure. In previous studies we found that peritoneal macrophages collected from CAPD patients during an episode of peritonitis, a major complication of CAPD, have undergone striking changes in the (in vitro) release of cyclooxygenase products (PGE_2, PGI_2 and TXA_2) and in the intracellular levels of the second messenger cAMP, in comparison with cells obtained during an infection-free period (3). We postulated, that, in view of the fact that PGE_2 and PGI_2 are known to have inhibiting effects on several macrophage functions via elevation of the second messenger cAMP, the diminished release of PGE_2 and PGI_2 , as found during peritonitis, would allow the macrophages to release an increased amount of the proinflammatory polypeptide interleukin-1 (IL-1) (11). To test this hypothesis, first the (in vitro) release of IL-1 from macrophages of infected and uninfected patients, was to be examined. As decribed in *chapter 5*, peritoneal macrophages from infected patients released an increased amount of IL-1ß indeed, on the condition that the cells were stimulated (in vitro) with LPS. Macrophages from infected and uninfected patients released similar amounts of IL-1, when they were cultured in the absence of exogenous LPS. Since in our previous studies on the release of prostaglandins, only the spontaneous release (i.e. without exogenous LPS) was determined, the findings on IL-1 prompted us to investigate the release of PGE_2 and PGI_2 when the peritoneal macrophages were additionally cultured with LPS. As described in *chapter 6*, stimulating the macrophages with LPS, revealed some remarkable facts. First, whereas spontaneous PGE_2 and PGI_2 release from peritonitis macrophages was decreased, LPS stimulated release of

PGE_2 was similar for peritonitis and infection-free macrophages. On the other hand, LPS did not induce an increase in PGI_2 release, neither from peritonitis macrophages nor from infection-free macrophages. In other words, PGI_2 release from macrophages of infected patients is decreased in comparison with the release from those of infection-free patients, whether or not the macrophages are stimulated with LPS. These findings indicate, that PGI_2 and PGE_2 play different roles in the regulation of macrophage functions. A distinct role for PGE_2 and PG_2 in the regulation of macrophage functions was also found in granulomatous inflammation (21). As described in *chapter 7*, release of TNFα, another cytokine with proinflammatory actions, is increased in macrophages from infected patients, provided that the cells are stimulated with LPS. These findings are in full agreement with those on IL-1. Finally, in order to examine whether exogenous or endogenous PGE_2 could influence IL-1ß and TNFα release from peritoneal macrophages, these cells were cultured with PGE_2 or the cyclooxygenase inhibitor indomethacin. As described in *chapter 8*, PGE_2 induced invariably a dose dependent inhibition of TNFα release, while incubation with indomethacin induced a two fold increase of TNFα. In this respect, similar results were obtained for infection-free and peritonitis macrophages. These findings provide conclusive evidence, that PGE_2 can regulate TNFα release. In contrast, PGE_2 had hardly any effect on IL-1ß release from macrophages of infected and infection-free patients. Indomethacin failed to induce an increase in IL-1ß release, indicating that IL-1ß release is not regulated by exogenous or endogenous PGE_2.

9.2. General discussion

After this outline of the findings described in the previous chapters, it will be discussed next, whether a connection between these results may be establisded and which relevance they may have for the acquisition of functional competence of peritoneal macrophages during infection. Peritoneal macrophages isolated from an infectious environment turned out to release more IL-1ß and TNFα, provided they were additionally stimulated (in vitro) with LPS. This finding implies that the macrophages have undergone changes in the infectious inflammatory environment i.e. in vivo, which come to light only following stimulation with LPS in vitro (fig. 1).

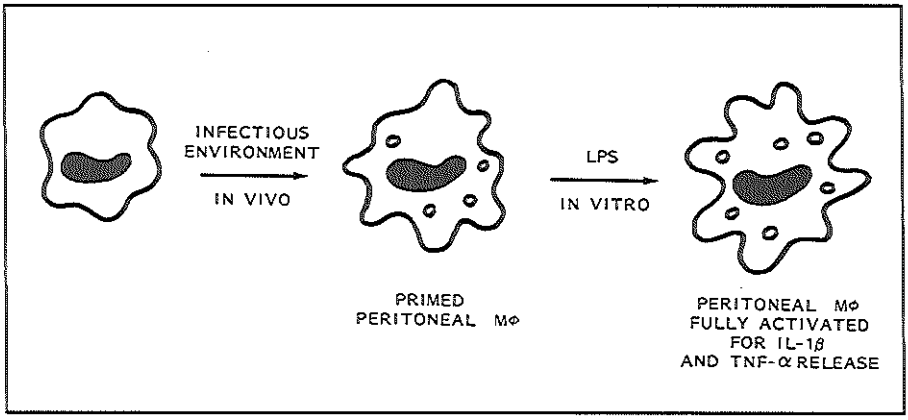

Fig.1 Hypothetical model for two step activation for IL-1ß and TNFα secretion by peritoneal macrophages during peritonitis. Peritoneal macrophages acquire full competence for IL-1ß and TNFα secretion during peritonitis in two stages. In vivo, i.e. in the infectious inflammatory environment, peritoneal macrophages which are monocytes recently migrated into the peritoneal cavity, are "primed" for secretion of IL-1ß and TNFα (first stage). The increased ability of these primed macrophages to secrete IL-1ß and TNFα is revealed solely, when they are additionally stimulated (in vitro) with LPS (second stage).

This is consistent with the concept put forward by Gordon, that macropahges acquire functional competence in a series of definite steps (12). Gordon et al. showed that macrophages harvested from mice after an intraperitoneal injection of LPS, released little plasminogen activator. Subsequent phagocytosis of latex or bacteria (in vitro) triggered these "primed" macrophages to release high levels of plasminogen activator as long as the phagocytic stimuli persisted. Similarly, in order to obtain full tumoricidal activity, macrophages need to be primed by lymphokines, while actual secretion of lytic substances is triggered by LPS (1,22). We found, that peritoneal macrophages from infected patients expressed enhanced antitumor activity (7). The concept of stepwise activation of macrophages appears to apply to the development of many functional responses (1). Recently much new information has become available on the complex molecular basis of macrophage activation (2,26). Among the many substances which are able to induce activation of macrophages, interferon γ (IFN-γ) remains the prototypic priming agent for various functional responses, including for the priming for the synthesis and release of IL-1 and TNFα. Considering, that lymphocytes are scant during

peritonitis (chapter 5), IFN-γ is not a likely candidate. Rather macrophage derived products, such as IFN-α, IFN-ß (27) and colony stimulating factors, or conceivably, PMN derived products might be considered as priming agents. At the molecular level, any substance which is able to affect processes prior to secretion, including transcription, translation and processing of the protein, could serve as priming signal. In addition to host derived products, cell wall components of gram positive bacteria, the majority of which were the causative microorganisms of peritonitis, can induce (in vitro) IL-1 and TNFα production.
Tissue macrophages were found to have a decreased capacity for IL-1 release, as compared to peripheral blood monocytes (28). Similarly, monocytes cultured in vitro, were found to have a decreased capacity for IL-1 release as compared with freshly isolated monocytes (5,9). Although the dwell time of peritoneal dialysate was the same for infected and infection-free patients, the possibility should be considered, that during peritonitis a more immature population of mononuclear phagocytes has migrated into the peritoneal cavity, which might have contributed to their increased capacity to release IL-1. On the other hand, TNFα is found to be released in similar amounts from monocytes and tissue macrophages (9). Moreover, in contrast to IL-1ß, TNFα is almost entirely released from the cell after biosynthesis. Consequently, the increased capacity of peritonitis macrophages to release TNFα, is likely to be entirely attributable to increased biosynthesis.

Role of prostaglandins in IL-1ß and TNFα release

As described in chapter 8, TNFα rather than IL-1ß release from peritoneal macrophages is suppressed by PGE_2. It is generally accepted however, that PGE_2 posttranscriptionally inhibits IL-1 release from mononuclear phagocytes. This is concluded from the observation that PGE_2 induces inhibition of IL-1 bioactivity in macrophage supernatants without concomitant decrease in IL-1mRNA accumulation (15,16). In our study, IL-1ß was determined using an immunoassay. We cannot rule out the possibility that the ELISA as used by us, detects also precursor IL-1ß. Therefore, it is conceivable, that the release of the mature, bioactive form of IL-1ß is inhibited by PGE_2. It was found by others, however, that PGE_2 has no effect on IL-1 synthesis by macrophages, but rather has a direct effect on thymocyte proliferation which was used as a measure for IL-1ß contents (13,20). Moreover, the possibility should be considered, that PGE_2

affects the release of IL-1 inhibitors like the IL-1 receptor antagonist. Anyway, our findings challenge the opinion, that PGE_2 acts, in general, as an inhibitor of IL-1 synthesis and release. Our finding, that PGE_2 suppresses TNFα release from macrophages agrees with what is found by others. Moreover, it is reported, that PGE_2 affects TNFα production at least at the level of transcription (23,24). TNFα and IL-1ß have many biological activities in common. Our finding, that their release from macrophages, which is a prerequisite for many biological activities is distinctly regulated by PGE_2, is in line with the finding of others concerning selective PGE_2 regulation of gene expression of these cytokines (23,25).
Now that the regulatory role of PGE_2 with regard to IL-1ß and TNFα release has been discussed, we revert to the postulate that the decreased secretion of PGE_2 by macrophages from peritonitis patients would allow these cells to release on increased amount of IL-1ß and TNFα, following stimulation with LPS. A decrease in the secretion of cyclooxygenase products was also found when human peritoneal macrophages were cultured in vitro with Staph. epidermidis (17). Our findings do not provide evidence, that PGE_2 regulates LPS induced IL-1 release. In contrast, LPS stimulated TNFα release is inhibited by PGE_2 so strongly, that the stimulatory effects of LPS were abolished completely at high doses of PGE_2. We found however that after stimulation with LPS, similar amounts of PGE_2 were released from macrophages of infected or infection-free patients. This raises the question, whether the decreased spontaneous (i.e. without exogenous LPS) PGE_2 release from peritonitis macrophages could have allowed these cells to release an increased amount of TNFα, as compared to infection-free macrophages, after stimulation with LPS. In light of the inhibitory effects of PGE_2 on the transcription of TNFα encoding gene, it is tempting to speculate that the decreased spontaneous release allows the peritonitis macrophages a start in the transcription rate, which is retained for any given endogenous or exogenous PGE_2 concentration after stimulation with LPS. On the other hand, it is the impaired release of PGI_2, that could have enabled the peritonitis macrophages to release more TNFα following stimulation with LPS, seeing that LPS stimulated macrophages from infected patients show also impaired PGI_2 release (fig. 2).

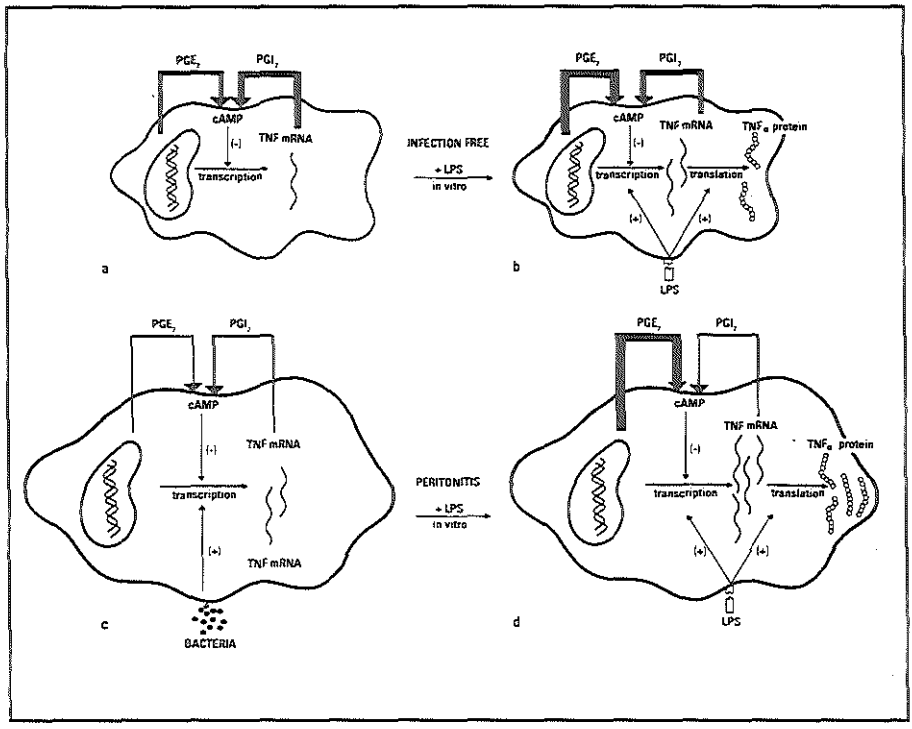

Fig. 2 Hypothetical model of the influence of peritonitis induced changes in prostanoid release on the release of TNFα from peritoneal macrophages. a) Peritoneal macrophages in an infection-free environment. b) Peritoneal macrophages stimulated in vitro with LPS after isolation from an infection-free environment. c) Peritoneal macrophages in an infectious environment (peritonitis). d) Peritoneal macrophages stimulated in vitro with LPS after isolation from an infectious environment. During peritonitis, TNFα gene transcription is stimulated by bacteria (mainly gram positive) (see c). The decrease in the release of PGE_2 and PGI_2 during peritonitis (c) as compared to an infection-free period (a), diminishes the suppressive effects of these prostanoids on TNFα gene transcription thereby further promoting TNFα gene transcription. These suppressive effects on PGE_2 and PGI_2 are mediated via intracellular cAMP elevation. When peritoneal macrophages are stimulated with LPS after isolation from the infectious environment (see d), both TNFα gene transcription and translation are stimulated. PGI_2 release from these macrophages (d) is decreased in comparison with PGI_2 release from LPS stimulated macrophages isolated from an infection-free environment (b). This may account for the fact that LPS stimulated TNFα release from peritonitis macrophages is increased in comparison with TNFα release from infection-free macrophages (b). The inhibitory effects of endogenously produced PGE_2 and PGI_2 on TNFα synthesis is further demonstrated by the finding that indomethacin further enhances LPS-stimulated TNFα release. (see chapter 8). Conceivably, also the putative increase in TNFα gene transcription of peritonitis macrophages in vivo (see c) may have contributed to increased TNFα synthesis by peritonitis macrophages after in vitro stimulation with LPS.

Macrophages are usually studied in chronic inflammatory processes, where mononuclear cells are the predominant cell type. Our studies may contribute to a deeper insight in the interactions of mediators with macrophages in acute, infectious inflammatory reactions. It should be mentioned however, that a sharp demarcation line between acute and chronic inflammations cannot be drawn always (8,29). Cytokines contribute to the process of eliminating microbial invaders. On the other hand, their action may be harmful, which is evident from the septic shock syndrome (19) and in arthritis (14), where these cytokines contribute to cartilage erosion and bone resorption. With regard to CAPD, better understanding of the release of cytokines, may be of relevance to the development of treatment modalities that either promote resistance against peritonitis of prevent complications such as sclerosing peritonitis.

9.3. Proposals for future research

The results as described in this thesis, strengthen our view that comparing peritoneal macrophages from infected CAPD patients with those from infection-free patients, provides an unique opportunity for investigating infectious-inflammatory processes in humans.
Furthermore, by comparing the release of and response to mediators synthesized by blood monocytes, peritoneal macrophages and macrophages isolated during an episode of peritonitis, we could gain a valuable insight into the biochemical and pharmacological aspects of differentiation and activation of macrophages.

1. *Comparison of infection-free and peritonitis macrophages*
a) A crucial question, directly following from our studies, is what are the (molecular) mechanisms behind "priming" of macrophages during an episode of infectious peritonitis. Does the increased capacity to secrete IL-1ß and TNFα during peritonitis reflect increased synthesis of either of these cytokines, or is solely the secretion increased in comparison with infection-free macrophages? If the synthesis is increased, it is relevant to know at what level this increased synthesis is regulated. Finding increased IL-1ß or TNFα mRNA levels in peritonitis macrophages, that are not stimulated with LPS, would indicate regulation at the level of transcription rate (or stability of mRNA).

Moreover stimulating macrophages from infection-free patients in vitro with bacteria or bacterial products, could provide more insight in the mechanisms of priming (17,18).
b) Recently, a mononuclear phagocyte derived IL-1 inhibitor, was characterized as a pure IL-1 receptor antagonist. (30) Now that immunoassays have become available, the role of this native IL-1 inhibitor in the regulation of IL-1 actions could be further investigated (6). Our model may provide a unique tool to study how the secretion of this native IL-1 inhibitor is regulated in acute inflammatory reactions.
Similarly, our model could be used to study the role of postulated soluble TNFα receptors as regulators of the actions of TNFα with regard to peritoneal macrophages from an infectious and infection-free environment (10).

2. *Comparison of peritoneal macrophages and peripheral blood monocytes*
Tissue macrophages, which are thought to originate from peripheral blood monocytes, have been found to have a decreased capacity to release IL-1 in comparison with their precursors (28). Comparison of peripheral blood monocytes and peritoneal macrophages with regard to the production of cytokines (e.g. IL-1) and other mediators, could afford an unique opportunity to study the alterations which monocytes undergo after their migration to the peritoneal cavity.

After having put forward some suggestions about future research, a more general question can be posed. Do the findings, as described in this thesis, offer prospects of therapeutical applications? In particular, are PGE_2 and cyclooxygenase inhibitors, such as indomethacin useful as therapeutical agents, viewing their potent actions on TNFα release? One would expect, for example, that PGE_2 could be beneficial in TNFα-mediated diseases such as septic shock. In many cases however, PGE_2 and TNFα act synergistically on target cells. It is tempting to speculate, that selective inhibition of TNFα secretion could be achieved by trapping PGE_2 in liposomes, which have the tendency to be accumulated in macrophages. (4) By simultaneously administering cyclooxygenase inhibitors, actions and synthesis of TNFα could be separately manipulated.

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Chapter 10

Samenvatting en discussie

10.1. Samenvatting

Continue Ambulante Peritoneale Dialyse (CAPD) is een vrij recente vorm van nierfunctievervangende behandeling, die steeds meer toepassing vindt. Eén van de belangrijkste complicaties van deze therapie is het optreden van een acute peritonitis (buikvliesontsteking), meestal veroorzaakt door microben (bacteriën, schimmels) die zich vanuit de omgeving toegang tot de buikholte hebben verschaft. Tijdens een peritonitis, is er in de buikholte een sterke toename van het aantal leucocyten, vooral van neutrofiele granulocyten, maar ook van macrofagen. Deze kunnen uit het dialysaat geïsoleerd worden, nadat dit na een verblijfstijd van 4-8 uur in de buikholte, is uitgelopen. Ook wanneer CAPD patiënten geen peritonitis hebben, kunnen macrofagen uit het dialysaat geïsoleerd worden, zij het in veel geringere aantallen. In dit proefschrift gaat het om de betekenis, die diverse door macrofagen geproduceerde mediatoren hebben m.b.t. de regulatie, d.w.z. versterking of juist afzwakking, van de acute onstekingsreactie, zoals die optreedt bij een infectie van de buikholte. Uit eerdere onderzoeken was ons gebleken, dat tijdens een episode van peritonitis, geïsoleerde macrofagen in vitro gemiddeld veel minder cyclooxygenase produkten (PGE_2, PGI_2 en TXA_2) afgeven dan macrofagen, die geïsoleerd werden bij afwezigheid van infectie (3). Ook het gehalte van cAMP in de macrofagen was veel lager tijdens peritonitis. Van PGE_2 en PGI_2 is het bekend, dat ze een remmende invloed kunnen uitoefenen op diverse functies van de in het afweerapparaat zo'n centrale rol spelende ontstekingscellen als de macrofagen (onstekingsremmende of wel antiinflammatoire effecten). Wij postuleerden toen, dat het feit, dat macrofagen tijdens een acute, door infectie veroorzaakte, ontstekingsreactie een verminderde hoeveelheid stoffen met een potentieel remmende invloed op macrofagen, produceren, de macrofagen in staat zou kunnen stellen een grote hoeveel-

heid interleukine-1 (IL-1) te maken, (11) een eiwit, dat na afgifte door de macrofaag overal in het lichaam vele proinflammatoire (=ontstekingsbevorderende) effecten uitoefent. Als deze hypothese juist zou zijn, dan zou, ten eerste de afgifte van IL-1 door macrofagen toegenomen moeten zijn, wanneer deze geïsoleerd worden tijdens peritonitis. Ten tweede zou aangetoond moeten worden, dat als macrofagen geïncubeerd worden met exogeen PGE_2 of PGI_2, dan wel met een cyclooxygenase syntheseremmer, deze respectievelijk een afname dan wel toename in de IL-1 afgifte zouden moeten laten zien. In *hoofdstuk 5* wordt beschreven, dat de peritoneale macrofagen van patiënten met peritonitis inderdaad meer IL-1 produceren, maar dan alleen indien ze in vitro gestimuleerd worden met LPS. Bij incubatie in afwezigheid van LPS blijken macrofagen van patiënten met peritonitis evenveel IL-1 te produceren als die van patiënten zonder infectie. Omdat in onze vroegere onderzoeken, de afgifte van cyclooxygenase produkten alleen de spontane afgifte was bepaald, d.w.z. zonder dat de macrofagen in vitro met LPS waren gestimuleerd, waren de resultaten over de IL-1 afgifte voor ons aanleiding om de afgifte van PGE_2 en PGI_2 te bepalen bij met LPS gestimuleerde macrofagen. Zoals in *hoofdstuk 6* staat beschreven, gaf dit onderzoek een aantal opmerkelijke bevindingen te zien. Terwijl evenals in de vroegere onderzoeken, de spontane afgifte van PGE_2 en PGI_2 door tijdens peritonitis geïsoleerde macrofagen verlaagd was, was de PGE_2 afgifte van met LPS gestimuleerde macrofagen hetzelfde voor macrofagen afkomstig van patiënten met en van patiënten zonder peritonitis. De PGI_2 afgifte daarentegen, reageerde in het geheel niet op LPS. Dit gold zowel voor macrofagen van patiënten met als voor macrofagen van patiënten zonder peritonitis. Anders gezegd, de afgifte van PGI_2 door tijdens peritonitis geïsoleerde macrofagen is veel lager dan de afgifte door macrofagen, die afkomstig zijn van patiënten zonder peritonitis, ongeacht of deze al dan niet door LPS gestimuleerd worden. Dit kan erop wijzen, dat PGE_2 en PGI_2 een verschillende regulerende invloed hebben op macrofagen, zoals reeds eerder was vastgesteld m.b.t. een granulomateuze onstekingsreactie (21). In *hoofdstuk 7* wordt beschreven, dat de afgifte van TNFα, een ander cytokine, dat veel proinflammatoire effecten met IL-1 gemeen heeft, eveneens is toegenomen in macrofagen van peritonitis patiënten, mits deze gestimuleerd worden met LPS. Dit is volledig in overeenstemming met de resultaten, die m.b.t. de IL-1 afgifte werden verkregen. Tenslotte moest onderzocht worden, in hoeverre PGE_2 betrokken is bij de regulatie van de afgifte van IL-1ß en TNFα. Hiertoe werden peritoneale macrofagen geïncubeerd in aanwezigheid van exogeen

PGE_2 of in aanwezigheid van de cyclooxygenaseremmer indomethacine. *In hoofdstuk 8* wordt beschreven, dat PGE_2 zonder uitzondering de TNFα afgifte op een dosis afhankelijke wijze remt, terwijl incubatie met indomethacine de afgifte deed verdubbelen. Deze resultaten werden verkregen zowel bij macrofagen van patiënten mèt als van patiënten zonder peritonitis. Hieruit blijkt duidelijk, dat zowel het exogeen toegediende als het door de macrofaag zelf geproduceerde PGE_2 betrokken is bij de afgifte van TNFα. Daarentegen bleek PGE_2 nauwelijks enig effect op de IL-1 afgifte van macrofagen te hebben. Evenmin nam de afgifte van IL-1 toe onder invloed van incubatie met indomethacine.

10.2. Discussie

Na dit overzicht, zal nu besproken worden in hoeverre er een verband gelegd kan worden tussen de in de vorige hoofdstukken beschreven resultaten. Ook zal ingegaan worden op de vraag, of de verkregen resultaten relevant zijn voor de wijze waarop macrofagen tijdens een infectie op hun taak worden voorbereid. Het bleek dus, dat macrofagen, die geïsoleerd werden vanuit een gebied waar een infectie is, meer IL-1ß en TNFα afgeven, mits ze (in vitro) met LPS werden gestimuleerd (fig. 1).

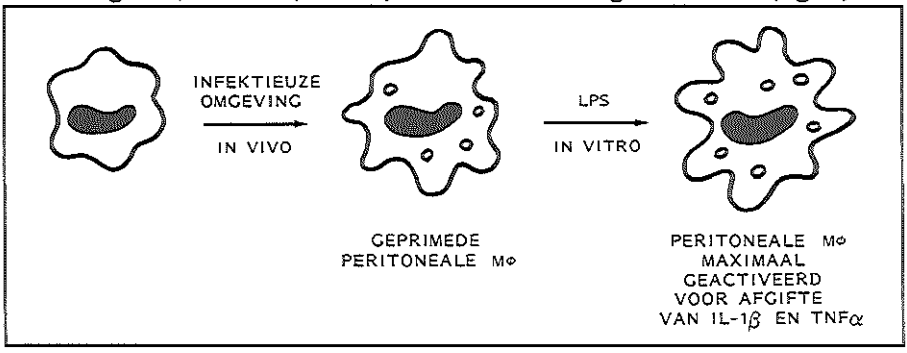

Fig. 1 Hypothetisch model, aan de hand waarvan wordt weergegeven hoe peritoneale macrofagen tijdens peritonitis in twee stappen activatie ondergaan voor de secretie van IL-1ß en TNFα. Peritoneale macrofagen verkrijgen hun vermogen tot maximale secretie van IL-1ß en TNFα in twee stadia. In vivo, waar zich de door infektie veroorzaakte ontstekingsreactie afspeelt, worden peritoneale macrofagen, die in feite recent naar de buikholte gemigreerde monocyten zijn, "geprimed" voor de secretie van IL-1ß en TNFα (1e stadium). Het toegenomen vermogen van deze "geprimede" macrofagen om IL-1ß en TNFα te secemeren, komt alleen aan het licht, als ze tevens (in vitro) met LPS gestimuleerd worden (2e stadium).

Dit houdt in, dat de macrofagen op de plaats van de infectie, dat wil zeggen in vivo, veranderingen hebben ondergaan, die pas aan het licht komen als ze (in vitro) met LPS worden gestimuleerd. Deze bevinding past goed in het concept, dat voor het eerst door Gordon en anderen is ontwikkeld, nl. dat macrofagen in een aantal stappen op hun functie worden voorbereid (12). Deze auteurs beschreven, dat peritoneale macrofagen van muizen, die geïsoleerd werden na een intraperitoneale injectie van LPS, slechts weinig plasminogeenactivator produceerden. Als de macrofagen evenwel (in vitro) aan fagocyterende prikkels werden blootgesteld (latex, of bacteriën), dan gingen deze door LPS "geprimede" macrofagen grote hoeveelheden plasminogeenactivator produceren zolang de fagocytose aanhield. Ook bij het verkrijgen van het vermogen om tumorweefsel te doden, dienen macrofagen eerst voorbereid ("geprimed") te worden door lymfokines, alvorens de afgifte van stoffen, die tumorweefsel kunnen doden, door LPS wordt bewerkstelligd (1,22). In dit verband kan opgemerkt worden, dat wij vonden dat van patiënten met peritonitis afkomstige macrofagen, een verhoogde anti-tumor activiteit bezitten (7). De opvatting, dat macrofagen stapsgewijs worden geactiveerd, is van toepassing op diverse functies van macrofagen (1). De laatste tijd is meer inzicht verkregen in de moleculaire mechanismen, die ten grondslag liggen aan de activatie van macrofagen (2,26). Van de vele stoffen, die een rol spelen bij de activatie van macrofagen is Interferon γ (IFN-γ) bij uitstek bekend vanwege zijn vermogen "priming" van macrofagen te effectueren, zoals bijv. de priming voor de synthese van TNFα en IL-1. Gezien het feit, dat lymphocyten sporadisch worden aangetroffen tijdens een peritonitis (hfdst. 5), komt IFN-γ niet in de eerste plaats in aanmerking. Veeleer dienen van macrofagen afkomstige stoffen, zoals IFNα en IFNβ (27) en CSF (colony stimulating factors) of wellicht, door neutrofiele granulocyten geproduceerde stoffen als kandidaten beschouwd te worden. Op het moleculaire vlak, kan elke stof, die in staat is om de aan de secretie voorafgaande processen te beïnvloeden, zoals de transcriptie, translatie en de "processing" van een eiwit, "priming" bewerkstelligen. Daarnaast zijn bepaalde bestanddelen van de wand van grampositieve bacteriën, die verantwoordelijk zijn voor het merendeel van de peritonitisepisoden, in staat (in vitro) de produktie van TNFα en IL-1 te bevorderen.

Er is vastgesteld, dat macrofagen t.o.v. monocyten een verminderde capaciteit tot de afgifte van IL-1 hebben (28). Hetzelfde geldt voor monocyten, die enige tijd in kweek gehouden zijn. Deze hebben eveneens een verminderde capaciteit tot IL-1 afgifte t.o.v. pas geïsoleerde monocyten

(5,9). Hoewel de verblijfstijd van het dialysaat in de buikholte van patienten met en zonder peritonitis hetzelfde is, moet het niet uitgesloten worden geacht, dat tijdens peritonitis een nog onrijpere populatie van mononucleaire fagocyten naar de buikholte is gemigreerd. Dit zou dan wellicht een bijdrage kunnen hebben geleverd aan de door ons waargenomen vermeerderde capaciteit voor IL-1 afgifte. Anderzijds wordt TNFα in vergelijkbare mate door monocyten en macrofagen afgegeven (9). Bovendien wordt TNFα in tegenstelling tot IL-1 na de synthese vrijwel geheel door de cel uitgescheiden. Het ligt daarom voor de hand om de toegenomen capaciteit voor de afgifte van TNFα, door tijdens peritonitis geïsoleerde macrofagen, geheel aan een toegenomen synthese toe te schrijven.

De rol van prostaglandines bij de afgifte van IL-1ß en TNFα

In hoofdstuk 8 werd m.b.t. peritoneale macrofagen aangetoond, dat het de afgifte van TNFα en niet die van IL-1ß is, die door PGE_2 wordt geremd. In het algemeen wordt echter de opvatting gehuldigd, dat PGE_2 de afgifte van IL-1 door monocyten en macrofagen onderdrukt en wel nadat transcriptie van het IL-1 gen heeft plaatsgevonden. Men heeft dit afgeleid van de waarneming, dat PGE_2 een afname van de bioactiviteit van IL-1 veroorzaakt, zonder dat dit gepaard gaat met een afname van IL-1 mRNA (15,16). In ons onderzoek is IL-1ß bepaald met behulp van een immunoassay (ELISA). Het is zeer wel mogelijk dat de door ons gebruikte ELISA, ook het "voorloper"-molecuul van IL-1ß, dat geen biologische activiteit heeft, herkent. Het is daarom niet uitgesloten dat PGE_2 de afgifte van alleen de vorm van IL-1ß met biologische activiteit, d.w.z. het "rijpe" IL-1ß remt. Er zijn evenwel ook onderzoekers, die voor de IL-1 bepaling van een bioassay (thymocyten proliferatie assay) gebruik hebben gemaakt en daarbij tot de conclusie zijn gekomen, dat PGE_2 niet de afgifte van IL-1 door de macrofagen remt, maar de thymocyten proliferatie, die gebruikt wordt als maat voor IL-1, direct beïnvloedt (1,20). Om de discrepantie tussen de IL-1ß bepaling met behulp van een bioassay en die met behulp van een immunoassay te verklaren, kan ook de mogelijkheid overwogen worden, dat PGE_2 de afgifte van door macrofagen geproduceerde stoffen, die de biologische activiteit van IL-1 remmen, zoals de recent ontdekte IL-1 receptor antagonist, beïnvloedt. Hoe dan ook, op grond van deze resultaten, kan de opvatting, dat PGE_2 in het algemeen fungeert als een remmer van de IL-1 afgifte, ter discussie worden gesteld. Wat TNFα betreft, is aangetoond, dat PGE_2 de synthese van dit cytokine door macrofagen remt

op het niveau van de transcriptie (23,24). IL-1ß en TNFα dienen eerst door de cellen te worden uitgescheiden alvorens zij het merendeel van hun biologische activiteiten, waarvan zij er vele gemeen hebben, kunnen uitoefenen. Onze bevinding, dat PGE_2 de afgifte van elk van beide cytokines op verschillende manier beïnvloedt, sluit aan bij wat anderen hebben gevonden met betrekking tot de wijze waarop PGE_2 de genexpressie van deze cytokines reguleert (23,25).

Nu de regulatie van de afgifte van IL-1ß en TNFα door PGE_2 is besproken, komen we nu terug op het eerder geformuleerde postulaat, dat de verminderde secretie van PGE_2 door macrofagen van patiënten met peritonitis, deze cellen in staat zou kunnen stellen meer IL-1 (en TNFα) uit te scheiden (na stimulatie met LPS). Ook stimulatie van peritoneale macrofagen, die van CAPD patiënten afkomstig zijn, met Staph. epidermidis in vitro, bleek een afname in de secretie van cyclooxygenase produkten te weeg te brengen (17). Onze resultaten pleiten er niet voor, dat PGE_2 de door LPS geïnduceerde afgifte van IL-1ß reguleert. Daarentegen wordt de door LPS geïnduceerde afgifte van TNFα zo sterk door PGE_2 onderdrukt, dat de stimulerende effecten van LPS geheel te niet gedaan worden bij hoge doses PGE_2. Echter bleken macrofagen van patiënten met en zonder peritonitis vergelijkbare hoeveelheden PGE_2 uit te scheiden, indien ze met LPS werden gestimuleerd. De vraag rijst dan of de verminderde spontane (d.w.z. zonder exogeen LPS) afgifte van PGE_2 door macrofagen van peritonitis patiënten een rol zou kunnen spelen bij het feit, dat macrofagen van peritonitis patiënten meer TNFα kunnen afgeven dan die van patiënten zonder peritonitis. Gezien de remmende invloeden van PGE_2 op de transcriptie van TNFα, is het verleidelijk om te speculeren, dat de verminderde spontane afgifte van PGE_2 de tijdens peritonitis geïsoleerde macrofagen een voorsprong geeft in de transcriptie snelheid van het TNFα gen, die zij houden bij iedere exogene of endogene PGE_2 concentratie na stimulatie met LPS. Maar het is ook mogelijk, dat het juist de verminderde afgifte van PGI_2 is, die een toename van de TNFα uitscheiding door macrofagen van peritonitis patiënten, mogelijk maakt, daar immers de tijdens peritonitis geïsoleerde macrofagen een verminderde PGI_2 afgifte te zien geven, ook na stimulatie met LPS (fig. 2).

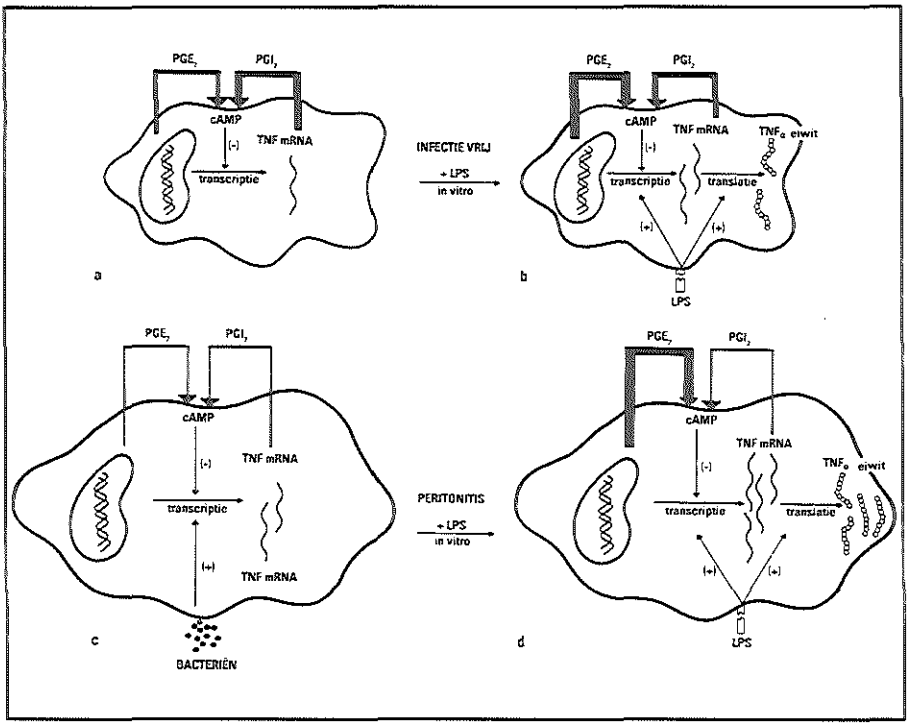

Fig. 2 In dit hypothetische model wordt weergegeven hoe de tijdens peritonitis opgetreden veranderingen in de afgifte van prostanoiden van invloed kunnen zijn op de secretie van TNFα door peritoneale macrofagen. a) Peritoneale macrofagen in een infektievrije omgeving. b) Peritoneale macrofagen, die in vitro worden gestimuleerd met LPS, na isolatie uit een infektievrije omgeving. c) Peritoneale macrofagen in een gebied met infektie (peritonitis) d) Peritoneale macrofagen, die in vitro met LPS worden gestimuleerd na isolatie uit een gebied met infektie (peritonitis). Tijdens peritonitis wordt de transcriptie van TNFα gestimuleerd door bacteriën (vnl. gram positieve) (zie c). De in vergelijking met een infektievrije periode (a) verminderde afgifte van PGE_2 en PGI_2 tijdens peritonitis (c), doet de remmende werking van deze prostanoïden op de transcriptie van TNFα afnemen, zodat de transcriptie van TNFα verder kan toenemen. Dit remmende effect van PGE_2 en PGI_2 wordt gemedieerd via een stijging van het intracellulaire cAMP-gehalte. Wanneer peritoneale macrofagen met LPS worden gestimuleerd, worden zowel de transcriptie als de translatie van TNFα gestimuleerd (b,d). Hierbij geven met LPS gestimuleerde macrofagen minder PGI_2 af wanneer ze afkomstig zijn van een gebied met infektie (d). Mogelijk kan dit dienen ter verklaring van het feit, dat van patiënten met een peritonitis afkomstige peritoneale macrofagen meer TNFα afgeven, (d), als ze met LPS worden gestimuleerd dan die van patiënten zonder infektie (b). De remmende werking van endogeen geproduceerd PGE_2 en PGI_2 op de TNFα synthese blijkt immers uit de bevinding dat indomethacine de door LPS gestimuleerde afgifte van TNFα verder doet toenemen (zie hfdst. 8). Het is evenwel ook mogelijk dat de veronderstelde toename in de transcriptie van TNFα tijdens peritonitis in vivo (zie c) er toe heeft bijgedragen dat met LPS (in vitro) gestimuleerde peritoneale macrofagen meer TNFα synthetiseren, wanneer ze afkomstig zijn uit een gebied met infektie (peritonitis) (d).

Macrofagen worden doorgaans onderzocht in verband met chronische ontstekingsprocessen, waar ze ook in aantal sterk vertegenwoordigd zijn. Onderzoek, zoals in die proefschrift beschreven, kan het inzicht verdiepen in de wisselwerking tussen cytokines en macrofagen in acute, door infectie veroorzaakte en door neutrofiele granulocyten gedomineerde ontstekingsreacties, waarbij dient te worden opgemerkt, dat acute en chronische ontsteking niet altijd scherp van elkaar te onderscheiden zijn (8,29). Cytokines als IL-1ß en TNFα kunnen een gunstige invloed hebben op de eliminatie van microorganismen. Maar zij kunnen ook schadelijke effecten hebben, zoals het geval blijkt bij het syndroom van de septische shock (19) en bij arthritis, waar ze kraakbeen erosie en botresorptie veroorzaken (14). Voor de CAPD zou een groter inzicht in de wijze waarop deze cytokines worden uitgescheiden, van belang kunnen zijn om behandelingsmethoden te ontwikkelen, die hetzij de weerstand tegen (infectieuze) peritonitis verhogen, hetzij complicaties als scleroserende peritonitis kunnen helpen voorkomen.

10.3. Voorstellen voor toekomstig onderzoek

De in dit proefschrift beschreven resultaten sterken ons in de mening dat door peritoneale macrofagen afkomstig van CAPD patiënten met een peritonitis te vergelijken met die afkomstig van patiënten zonder infectie, een unieke mogelijkheid wordt verkregen om bij de mens acute door infectie veroorzaakte ontstekingsreacties te onderzoeken. Verder zou ons inzicht in de biochemische en farmacologische aspecten van differentiatie en activatie van macrofagen verdiept kunnen worden door monocyten van het perifere bloed, peritoneale macrofagen en tijdens peritonitis geïsoleerde macrofagen vergelijkenderwijs te onderzoeken op hun afgifte van en reactie op mediatoren.

1) *Vergelijking van tijdens een peritonitis en infectievrije periode geïsoleerde macrofagen*

a) een vraag, die direct opkomt, is wat de mechanismen zijn (bijv. op het moleculaire vlak) van de "priming" van de macrofagen tijdens peritonitis. Hangt de toegenomen capaciteit om IL-1ß en TNFα uit te scheiden samen met een toegenomen synthese van IL-1, of is uitsluitend de secretie zelf toegenomen? Zou er sprake blijken te zijn van een toename in de synthese,

dan is het van belang te weten, op welk niveau deze toegenomen synthese wordt geregeld. Als er in niet met LPS gestimuleerde macrofagen van peritonitis patiënten een toename van mRNA van IL-1ß of TNFα zou worden gevonden, dan zou dit er op kunnen duiden dat de "priming" berust op een toegenomen transcriptie.
Verder zou in vitro onderzoek, waarbij macrofagen in vitro worden geincubeerd met bacteriën (of bacteriële produkten), het inzicht in de mechanismen van "priming" bij infecties kunnen vergroten (17,18).
b) Onlangs werd een zuivere IL-1 receptor antagonist beschreven, die geproduceerd en uitgescheiden wordt door mononucleaire phagocyten (30). Nu immunoassays ontwikkeld zijn, zou de rol die deze natuurlijke receptor blokkeerder van IL-1 in de regulatie van de biologische activiteiten van IL-1 speelt, verder onderzocht kunnen worden (6). Onderzoeken, zoals die door ons beschreven zijn, bieden een unieke mogelijkheid de regulatie van de secretie van deze natuurlijke IL-1 remmer tijdens ontstekingsreacties te bestuderen. Ook zou de rol van oplosbare TNFα receptoren, waarvan verondersteld wordt dat deze de werking van TNFα kunnen reguleren bestudeerd kunnen worden. Tijdens een peritonitis en een infectievrije periode geïsoleerde macrofagen zouden vergeleken kunnen worden op hun (eventuele) secretie van deze oplosbare TNFα receptoren (10).

2. Vergelijking van peritoneale macrofagen en monocyten.
Er is gebleken, dat macrofagen, die van monocyten afkomstige cellen zijn, in vergelijking met monocyten een verminderde capaciteit hebben voor de afgifte van IL-1 (28). Het vergelijken van monocyten en peritoneale macrofagen wat betreft de produktie van cytokines en andere mediatoren, biedt een unieke gelegenheid om de veranderingen die monocyten ondergaan nadat ze naar de buikholte zijn gemigreerd, te bestuderen.

Na een aantal suggesties te hebben gedaan voor toekomstig onderzoek, kan tenslotte nog de volgende vraag worden gesteld. Bieden de in dit proefschrift beschreven resultaten uitzicht op therapeutische toepassingen? Meer in het bijzonder, kunnen PGE_2 en cyclooxygenaseremmers als indomethacine therapeutische toepassing vinden gezien hun sterke invloed op de afgifte van TNFα? Het ligt voor de hand te veronderstellen dat PGE_2 bij de behandeling van de septische shock, waar TNFα een grote rol speelt, een gunstig effect zou kunnen hebben. In veel gevallen versterkt PGE_2 echter de werking van TNFα. Het is verleidelijk om te speculeren, dat een

selectieve remming van de secretie van TNFα zou kunnen worden bereikt, door PGE_2 op te sluiten in liposomen, die zich bij voorkeur in macrofagen ophopen (4). Als dan tegelijkertijd cyclooxygenaseremmers (niet in liposomen ingesloten) toegediend zouden worden, zouden de afgifte van TNFα en de werking van TNFα op de cel apart beïnvloed kunnen worden.

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Curriculum Vitae

Marien Willem Johan Adriaan Fieren was born in Utrecht on July 7th, 1945. From 1957 he received secondary education at the "Christelijk Gymnasium" in Utrecht, where he graduated ("Gymnasium ß") in 1963. In the same year he commenced studies in medicine at the State University of Utrecht, where he received his MD ("artsexamen") in 1972. After having done the military service as a medical officer, he worked at the department of Internal Medicine of the St. Joseph Hospital in Kerkrade, the Netherlands. In 1975 he commenced training in internal medicine at the "Catharina Hospital" in Eindhoven under supervision of prof. dr. J.A. Flendrig. In 1979 he joined the department of Internal Medicine I (head: prof. dr. J. Gerbrandy), division of Nephrology (Prof. dr. L.D.F. Lameier), of the University Hospital "Dijkzigt", Erasmus University Rotterdam, the Netherlands. In 1980 he was recorded in the register of recognized specialists of the Netherlands Medical Association for the specialism of Internal Medicine. Presently he works as member of the senior staff of the department of Internal Medicine I. (head of the department: prof. dr. M.A.D.H. Schalekamp). As from may 1991 he is entered in the register of the Dutch Internists Association for the area of specialization of Nephrology.