Neutrophil endothelial cell interaction : investigations on TNF-[alpha] and E-selectin

NEUTROPHIL ENDOTHELIAL CELL INTERACTION

INVESTIGATIONS ON TNF-α AND E-SELECTIN

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HET IN DIT PROEFSCHRIFT BESCHREVEN ONDERZOEK WERD FINANCIEEL MOGELIJK GEMAAKT DOOR STEUN VAN DE NIERSTICHTING NEDERLAND.

"A tree is best measured when it is down"

(motto opera 'The civil wars', Robert Wilson and Philip Glass)

Opgedragen aan Hetty Praagman en Jan van der Horst

CONTENTS

ABBREVIATIONS USED

Ab	antibody
Ag	antigen
BCS	bovine calf serum
BSA	bovine serum albumin
BPI	bactericidal/permeability increasing protein
CD	cluster of definition
ELAM-1	endothelial leukocyte adhesion molecule-1 (also called E-selectin)
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
FCS	fetal calf serum
FMLP	n-formyl Methionyl Leucyl Phenylalanine
GM-CSF	granulocyte macrophage colony stimulating factor
ICAM	inter-cellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
h	human
HUVEC	human umbilical vein endothelial cell(s)
HI	heat inactivated
HS	human serum
LBP	LPS binding protein
LM	light microscopy
LPS	(bacterial) lipopolysaccharides
mAb	monoclonal antibody
PAF	platelet activating factor
PBS	phosphate buffered saline
PECAM-1	platelet endothelial cell adhesion molecule
PHA	phyto haem agglutinin
PMA	phorbol myristate acetate
PMN	polymorphonuclear leukocytes
r	recombinant
RCEC	renal cortical eptithelial cell(s)
RPMI	named a specific tissue culture medium
RT	room temperature
SD	standert deviation
T/E	targetcell / effectorcell
TNF	tumor necrosis factor-α

Chapter 1

INTRODUCTION

General introduction

As observed more than 100 years ago by Cohnheim using intravital microscopy (1), neutrophils begin to interact with the vessel wall by rolling along the endothelium, within minutes after injury to adjacent tissue. During this process, the velocity of rolling neutrophils is much lower than cells just tumbling in a shear flow near the vessel wall, suggesting increased adhesive interactions which partly resist the flow induced shear stress. On the basis of this observation, Cohnheim postulated molecular changes in vessel endothelium induced by inflammatory mediators leaking from the inflamed tissue.

During the last decade, three families of adhesion receptors participating in neutrophil interaction with endothelium have been defined: the integrin, immunoglobulin-related, and selectin molecules. During the same period of time, a series of novel soluble inflammatory mediators has been identified. These mediators, which are called cytokines, are produced locally, and have a mainly local function. The recent knowledge on adhesion molecules and cytokines has led to clear progress in understanding the regulation of neutrophil rolling along the vessel wall, and the subsequent steps of neutrophil behaviour during ongoing inflammation: neutrophil attachment to the vessel wall, neutrophil emigration into the tissue, chemotactic migration to the inflammatory site, and the activation of the toxic potential of neutrophils. Different aspects of the regulation of neutrophil behaviour during inflammation, and the identification of cytokines and adhesion molecules involved in it, are presented in this thesis. In this introduction an attempt is made to summarize and interpret the information now available on adhesion molecules and cytokines in terms of their specific relevance for neutrophil behaviour during inflammation.

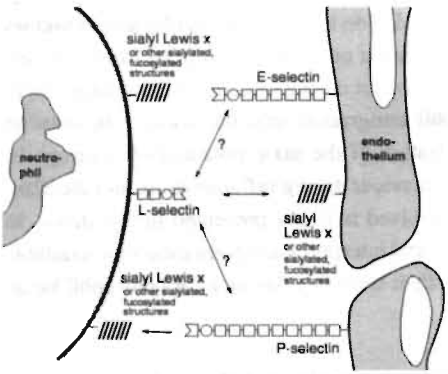
Chapter A of this introduction focusses on adhesion molecules and their role in neutrophil rolling, neutrophil attachment to the vessel wall and neutrophil emigration into the tissue. Chapter B focusses on the two responses of neutrophils to activation by inflammatory agents, neutrophil chemotaxis and neutrophil toxicity, and on the role of adhesion molecules in these two functions. Chapter C focusses on differences between the cytokine tumor necrosis factor-α (TNF) and other neutrophil activating agents, in their role in neutrophil behaviour.

Chapter A

ADHESION MOLECULES AND THEIR ROLE IN NEUTROPHIL ENDOTHELIAL CELL INTERACTION

A1. Integrins and immunoglobulin-related molecules

Integrins (integral membrane glycoproteins) form a heterogenic group of dimer molecules, composed of an α-chain and a B-chain, which are involved in many cell-cell and cell-substratum interactions (reviewed in 2). Three integrins, all sharing the same β-chain, are expressed on neutrophils: the $\alpha_M\beta_2$integrin, known as CD11a/CD18 or LFA-1, the $\alpha_L\beta_2$ integrin, known as CD11b/CD18 or Mac-1 and the $\alpha_X\beta_2$ integrin, known as CD11c/CD18. The integrin CD11a/CD18 binds to the immunoglobulin-related molecules ICAM-1 or CD54, ICAM-2 or CD102, and ICAM-3 or CD50 on endothelium and leukocytes; CD11b/CD18 binds to ICAM-1 but not to ICAM-2 (3-7), has affinity for Arg-Gly-Asp containing proteins such as C3bi and fibrinogen, for microbial surface components and for plastic (8-10); CD11c/CD18 is expressed in low amounts on neutrophils and has no clear function in neutrophil adhesion. The term β_2 integrin which is used in this introduction refers to CD11a/CD18 and CD11b/CD18 since little is known on the function of CD11c/CD18.



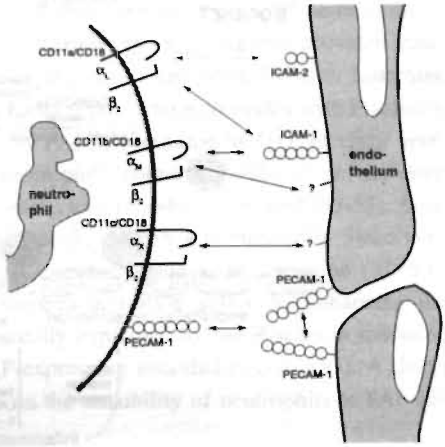
Integrins and immunoglobulin-related molecules

The three ICAM's (InterCellular Adhesion Molecules) are more closely related to each other than these molecules are to other immunoglobulin super-family members (5, 11, 12). ICAM-1 and ICAM-2 are expressed on endothelium, on mononuclear leukocytes, but not on neutrophils, whilst ICAM-3 is absent on endothelium, but is highly expressed by resting leukocytes including neutrophils. The three ICAM's participate in adhesive reactions of multiple cell types (3, 6, 7, 13).

PECAM-1 (Platelet Endothelial Cell Adhesion Molecule 1) or CD31 is another immunoglubilin superfamily member, expressed by platelets, T-cells, mononuclear cells and neutrophils, and is concentrated in the junctions of endothelial cells (14, 15). PECAM-1 has not been shown to bind to other adhesion molecules. PECAM-PECAM interactions might mediated both homotypic and heterotypic adhesive events (16-18).

A2. Selectins

The selectins are the most recently recognized class of adhesion molecules (19). They have an N-terminal lectin domain, one epidermal growth factor-like module, and from two to nine short consensus repeats, related to those found in complement-binding proteins. By contrast to integrins and immunoglobulin-related molecules, selectins have been found only on circulating cells and the endothelium, suggesting that they may be specialized for interactions within the vasculature.

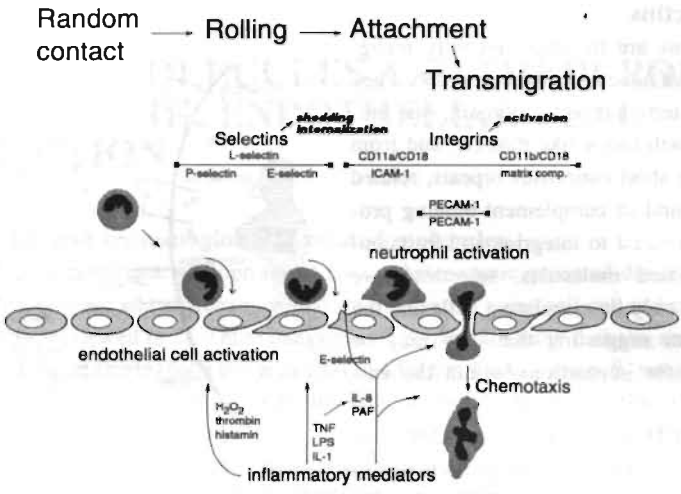


Selectins

P-selectin (CD62P, PADGEM or GMP-140) is expressed in α-granules of platelets and in Weibel-Palade bodies of endothelial cells, and is mobilized to the plasma membranes of these cells after activation by mediators of inflammation and hemostasis, allowing these cells to bind neutrophils and monocytes at the site of tissue injury (20-23). E-selectin (ELAM-1 or CD62E) is synthesized by endothelial cells in response to inflammatory mediators and promotes adhesion of neutrophils, monocytes, and a sub-population of lymphocytes (24-27). L-selectin (LAM-1, LECAM-1, or CD62L) is expressed on leukocytes, facilitates lymphocyte binding to endothelium during recirculation through peripheral lymph nodes, and mediates neutrophil emigration at inflammatory sites (28, 29). Specific partially overlapping carbohydrate ligands for selectins have been defined, such as sialyl Lewis x or CD15s on glycolipids and on the termini of N- and O-linked oligosaccharides (30-32). Interestingly, L-selectin on neutrophils, but not on lymphocytes, carries sialyl Lewis x carbohydrate groups (33), and plays a prominent role in neutrophil binding to E-selectin and P-selectin on endothelium (33-34)

A3. Selectin molecules mediate neutrophil rolling

Binding of β_2 integrins to ICAM's, and selectin mediated binding facilitate different forms of neutrophil endothelial cell interactions. Neutrophil rolling on endothelium at venous shear stress can be induced solely by *de novo* expression of P-selectin and E-selectin on endothelial cells (35-38). In contrast to neutrophil adhesion at static conditions, neutrophil rolling can not be induced by neutrophil activation. Moreover, neutrophil activation decreases the efficiency of adhesion to P-selectin (37) and to E-selectin (34). Endothelial cell exposure to thrombin, H_2O_2, or histamine induces P-selectin mediated neutrophil rolling within minutes, whilst E-selectin mediated neutrophil rolling peaks after 2 to 6 hours of endothelial cell activation with TNF, IL-1β or LPS (36, 37). In absence of additional neutrophil activation, rolling neutrophils remain round, and do not attach to the endothelium or emigrate (37).



The regulation of neutrophil rolling and attachment

A4. β2 integrin molecules mediate neutrophil attachment in response to neutrophil activation

As inflammatory reactions proceed, neutrophil rolling, as observed by intravital microscopy, decreases in velocity and is interrupted by halts until neutrophils come to a firm stop (1). When neutrophils are activated they first undergo a bipolar shape change which can even be induced when cells are held in suspension (37), and is also apparent in β_2 integrin deficient-neutrophils (39). Rolling neutrophils, or neutrophils which contact a surface in the absence of shear stress subsequently become attached to the surface, and flatten slightly. Neutrophil attachment is mediated by an increase in the avidity of the neutrophil β_2 integrins CD11a/CD18 and CD11b/CD18, which is initiated within minutes after neutrophil activation (40-43) and appears analogous to an increase in avidity described for CD11a/CD18 in T lymphocytes in response to antigen receptor cross-linking (44, 45). Under venous shear stress, activation of neutrophils is insufficient to trigger neutrophil attachment, since initial β_2 integrin interaction with ICAM-1 or other ligands is not shear stress resistant (36, 46). In contrast to selectin mediated adhesion, β_2 mediated adhesion is dependent on temperature, ATP, and presence of Mg^{++} (47, 48). When activated neutrophils are allowed to attach and flatten via β_2 integrin dependent mechanisms for several minutes, attachment can increase strongly and become resistant to extreme shear stresses (37, 49).

A5. β_2 Integrin activation during neutrophil interaction with endothelium activated with P-selectin inducing agents

During the interaction of rolling neutrophils with endothelium, activated either with P-selec-

tin or with E-selectin inducing agents, the neutrophil β_2 integrin CD11b/CD18 is'activated'; it acquires increased adhesive activity, which strengthens the attachment of neutrophils to activated endothelium. Different factors seem to coöperate in triggering this activated state. Activation of endothelium with the P-selectin inducing agents thrombin, H_2O_2, or histamine induces surface expression of platelet activating factor (PAF) which coincides with P-selectin expression (50-53). When neutrophil activation by PAF is prevented by PAF receptor antagonist or other means, β_2 integrin activation by neutrophils, contacting endothelium activated by P-selectin inducing agents is prevented completely, and adhesion is reduced (53-55). Antibodies reactive with P-selectin, however, also reduce β_2 integrin activation (53). Since neutrophil binding to P-selectin alone is insufficient to induce β_2 integrin activation (37, 53), P-selectin is thought to effect neutrophil β_2 integrin activation solely by enhancing the response of neutrophils to PAF. This can be partially explained by the P-selectin mediated increased contact between neutrophils and PAF-expressing endothelial cells (53). A direct effect of P-selectin interaction with neutrophils on the sensibility of neutrophils to PAF has also been described (53).

A6. β_2 Integrin activation during neutrophil interaction with endothelium activated with E-selectin inducing agents

In parallel to endothelial cell activation with P-selectin inducing agents, activation of endothelium with the E-selectin inducing agents TNF, IL-1β, and LPS triggers PAF surface expression, which peaks together with E-selectin expression, after 2 to 6 hours of activation (56, 56b, 56c). In contrast to neutrophil adherence to endothelium activated with P-selectin inducing agents, neutrophil adherence to E-selectin expressing endothelium is not reduced by addition of PAF-receptor antagonists (48, 56b). Additional involvement of other factors might make the role of PAF in β_2 integrin activation less essential. IL-8 and E-selectin itself could be such factors. In contrast to endothelial cells activated with P-selectin inducing agents, endothelial cells activated by E-selectin inducing agents will synthesize and release IL-8, a known neutrophil chemo-attractant (reviewed in 57). IL-8 alone is sufficient to induce β_2 integrin activationand can therefore increase neutrophil adhesiveness (58, 59).

In addition to the effect of IL-8 on neutrophils, E-selectin-ligand interactions seem to have a more clear direct effect on neutrophils than P-selectin-ligand interactions. E-selectin can, even in a purified form, trigger activation of neutrophil $\beta_2$integrins (48, 60). Three additional pathways, involving PAF, IL-8 and E-selectin itself, thus might cooperate in inducing activation of β_2 integrins of neutrophils contacting E-selectin expressing endothelium.

A7. Modulation of neutrophil binding to E- and P-selectin as a specialized function enabling neutrophil rolling and extravasation

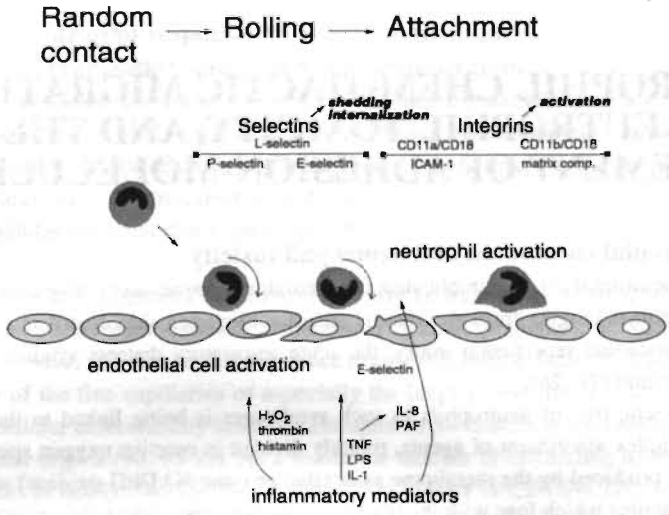
Neutrophil migration is thought to be based on variation in the number or the affinity of binding sites for neutrophils. Neutrophils migrate to the site with maximal binding capacity (61). Interestingly, the high affinity interactions between neutrophils and selectin molecules seem to result in a short-time contact, followed either by rolling downstream or by neutrophil

extravasation (chapter A8). A decrease in affinity of selectin-ligand binding rapidly upon attachment, could be an explanation for such behaviour, which would overcome the requirement of stronger binding sites at the leading front of cell motility. A decrease in affinity of neutrophil binding to E-selectin (34) and P-selectin (37) has been described to occur directly after neutrophil activation. This might be explained by shedding of the E-selectin and P-selectin ligand L-selectin from the neutrophil surface within minutes after activation (34, 62-65) and by the reduction in the amount of the neutrophil selectin ligand sialyl Lewis-x on other surface structures than L-selectin after neutrophil activation (33).
These observations are consistent with a model of neutrophil extravasation in which initial selectin mediated shear stress resistent attachment will bring neutrophils in closer contact to, and prolong their interaction with inflammatory mediators such as PAF and IL-8 originating from activated endothelium and the surrounding tissue. In addition, selectin-ligand binding can activate neutrophils directly (48, 53, 60). In response to this activation, selectin mediated adhesion is reduced and a more delicately regulated, β_2 integrin mediated adhesion pathway is activated, enabling directed (trans)migration. Whether the reduction in L-selectin and sialyl Lewis-x can occur focally within seconds at the site of neutrophil-endothelial cell contact, resulting in local disconnection if simultaneous activation of β_2-integrin avidity has been insufficient, and thus leading to the characteristic rolling behaviour, remains to be elucidated.

In situations of severe systemic inflammation, presence of circulating neutrophil activating factors could induce L-selectin shedding of circulating neutrophils and therefore down-regulate selectin mediated neutrophil endothelial cell interaction. This mechanism might limit neutrophil extravasation in situations where circulating E-selectin inducing agents such as LPS and TNF cause systemic E-selectin expression.

A8. Neutrophil emigration from the vasculature

Neutrophils appear to reach the point at which they emigrate by rolling; no active migration along the vessel wall is evident by intravital microscopy. While the cell is flattening, a pseudopod is extended through the vessel at a junction between the endothelial cells. Transmigration continues as the pseudopod grows in ramifications and size until the entire cell body has emerged through a narrow gap between the endothelial cells (1, 66), a process which is clearly distinct from *trans-cellular* passage of mature blood cells from bone marrow tissue into marrow sinuses (67).
These observations have been made long before the relation between neutrophil rolling and E-selectin or P-selectin expression became apparent. Although the capacity of E-selectin and P-selectin to mediate neutrophil rolling appears identical, their is a clear difference of E-selectin and P-selectin expressing endothelium in triggering neutrophil emigration. Endothelium activated with E-selectin inducing agents acquires polar characteristics. Efficient vectorial neutrophil migration from the luminal to the basal side of E-selectin expressing endothelial cell monolayers monolayer is evident (35, 36, 68, 69). Migration of neutrophils through E-selectin expressing endothelium requires interaction between β_2 integrins and ICAM-1(36,



The regulation of neutrophil rolling, attachment, and trans migration

68, 70). Although E-selectin plays a role in initial contact of neutrophils to such activated endothelium, neutrophil migration occurs independently from E-selectin (36, 68, 70, 71). After rapid activation of endothelial cell adhesiveness for neutrophils by P-selectin inducing agents, neutrophil migration through the endothelial cell monolayer is relatively low in comparison to E-selectin expressing endothelium (35, 72, 73). A difference between E-selectin expressing endothelium and P-selectin expressing endothelium is, that P-selectin expressing endothelium does not release the neutrophil chemotaxin IL-8 (57). IL-8 was indeed found to be responsible for neutrophil migration through endothelium activated with E-selectin inducing agents by forming a chemotactic gradient across endothelial cell monolayer which guides neutrophil migration to the basal side of the monolayer at which the highest IL-8 concentration exists (56, 62). Additional roles in mediating neutrophil transmigration have been claimed for endothelial cell associated PAF (56), and for the immunoglobulin related adhesion molecule PECAM-1 (17) which is concentrated at junctions between endothelial cells, but is also present on neutrophils. Besides inducing β_2 integrin activation (16, 17), involvement of direct adhesive PECAM-PECAM interactions has also been hypothesized (17).

Neutrophil transmigration through activated endothelium is not associated with endothelial cell damage or increased vascular permeability (35, 69, 74). Neutrophil degranulation and respiratory burst requires additional neutrophil activation (35, 74-76).
The regulation of the switch from neutrophil (trans)migration to neutrophil toxicity is discussed in part B of this introduction.

Chapter B

NEUTROPHIL CHEMOTACTIC MIGRATION AND NEUTROPHIL TOXICITY, AND THE INVOLVEMENT OF ADHESION-MOLECULES

B1. Neutrophil chemotaxis and neutrophil toxicity

The human neutrophil is being implicated as a mediator of tissue destructive events in inflammatory syndromes ranging from rheumatoid arthritis, blistering skin disorders and ulcerative colitis to myocardial reperfusion injury, the acute respiratory distress syndrome and acute allograft rejection (77, 78).

The pathogenetic role of neutrophils in such syndromes is being linked to their ability to release a complex assortment of agents, roughly divided in reactive oxygen species (metabolites of O_2^-, produced by the membrane associated enzyme NADPH-oxidase) and proteases (stored in granules which fuse with the plasma-membrane upon adequate activation). Reactive oxygen species and proteases are generally released in parallel, and cooperate in destroying cells and dissolving connective tissues (reviewed in 79).

Although these toxins normally defend the host against invading microbes, the neutrophil has little intrinsic ability to differentiate between foreign and host antigens and relies on other arms of the immune-system to select its targets. Besides recognizing surface bound complement and antibody Fc-parts, neutrophil-toxicity can be specifically triggered by a number of pro-inflammatory signal-molecules. A large number of such neutrophil agonists have been described, including microbial products such as bacterial lipopolysaccharides (LPS), the n-formylated oligopeptide FMLP (used as a model for physiologic, phlogistic peptides released from bacteria (80) and damaged mitochondria (81), and zymosan (baker's yeast ghost cells); lipid mediators such as PAF, leukotriene B_4 and monohydroxy-icosatetraenoic acids; cytokines such as TNF, GM-CSF, IL-1, and IL-8; and other inflammatory products such as complement factor C5a and immune complexes (reviewed in 82).

Agonist activation of neutrophils regulates two neutrophil functions: directing the neutrophil to the inflammatory scene, and activating neutrophil toxicity. Most soluble neutrophil agonists are chemotactically active, whilst others (TNF, LPS, GM-CSF) lack this capacity, and specifically activate neutrophil toxicity (chapter C). Neutrophil chemotaxis and neutrophil toxicity will be discussed and related to a number of more specific phenomena, seen as a response of neutrophils to agonist activation: changes in the neutrophil cytoskeleton, neutrophil sequestration in the fine capillary bed, β_2 integrin activation and rapid temporal respiratory burst activity. Neutrophil respiratory burst activity (resulting the production of reactive oxygen species) is used as a representative of neutrophil toxicity.

B2. Rapid neutrophil responses to agonist activation; Actin reorganization and neutrophil sequestration

Resting neutrophils are round and actin, one of the main cytoskeletal proteins, is distributed diffusely throughout the cell in its globular form. Within one minute after chemotaxin addition, the neutrophil surface begins to ruffle, and actin is cross-linked and converted to subcortical filamentous actin (F-actin), forming a cytoskeletal network (83-88). As a result of F-actin formation, cellular deformability is decreased (89, 90).

This rapid neutrophil responses can have impressive consequences *in vivo*, which are seen when chemotaxins are administrated intravenously in healthy animals, or when neutrophils are activated *ex vivo*, and then re-infused. Since neutrophils have a diameter of about 7μm and the diameter of the fine capillaries of especially the lungs is less than 6,5 μm (91, 92), the decrease in cellular deformability leads to neutrophil-sequestration in the capillary bed of the lungs and other organs (90, 93-95). As a result, the number of circulating neutrophils drops within minutes to nearly 0% (95-100). Although the affinity of CD11b/CD18 is increased in parallel to the F-actin mediated decrease in neutrophil deformability (40-43), CD11b/CD18 mediated adhesion does not have a clear role in neutrophil sequestration (90, J.M. Harlan, unpublished observation). Neutrophil emigration from the vasculature or induction of tissue-injury remains largely absent (97, 99) and within 15 to 30 minutes the number of circulating neutrophils returns to (supra) normal levels (95, 97-100).
Whether neutrophil sequestration has any physiological relevance is doubtful. During most inflammatory diseases, appearance of circulating neutrophil activating factors will be gradual, allowing adaptation of the sensitivity of neutrophils to agonist-stimulation (chapter B4 and B6). Neutrophil sequestration might be an over-reaction caused by the absence of sufficient time to allow such an adaptation.

B3. Rapid neutrophil responses to agonist activation; respiratory burst activity

Neutrophils exposed to high amounts of chemotaxins show nearly immediate respiratory burst activity. Depending on the type and the magnitude of agonist stimulation, reactive oxygen production can be measured which decreases after 3 to 30 minutes (87, 101-106). The direct respiratory burst in response to chemotaxins occurs independent from adherence; neutrophils in contact with a substrate for adherence and suspended neutrophils show similar initial responses (106, 107). Although transient CD11b/CD18 upregulation (103) and transiently increased adherence (108, 109) mediated by CD11b/CD18 (108) can be measured during this phase, CD11b/CD18 mediated substrate interaction is not essential for the toxic response of neutrophils in this phase (104, 110).

B4. Slow neutrophil responses to agonist activation; neutrophil chemotaxis

β_2 Integrin dependent neutrophil spreading in absence of shear stress, general membrane ruf-

fling, subcortical F-actin distribution and respiratory burst, seen within one minute after neutrophil-activation, are generally transient. Depending on the magnitude, and on the type (chapter C1) of the agonist to which the neutrophil is exposed, these response can last from a few minutes to approximately half an hour. After this time, the cells withdraw most ruffles, change from a stretched to a more rounded polarized morphology with ruffles only at the leading edge of the cell, and begin to migrate (84-86). In parallel, F-actin is redistributed to the leading edge of the cell (85, 111). Seizing of respiratory burst if neutrophils start migrating is not well documented. If neutrophils are subsequently exposed to higher agonist concentrations, neutrophils again stop migrating, round up and stretch temporarily (84). When agonist-concentrations are decreased, neutrophils also stop migrating and round up, but do not stretch (84).

Random migration changes to chemotaxis when neutrophils are placed in a gradient. Adhering neutrophils can orient and migrate in a gradient of FMLP, if a concentration-difference of 1% or more over their 10μm diameter exists (112, 113). Highest accuracy is reached at concentrations close to the dissociation constant, K_d, of FMLP from the cell receptor; at lower but also at higher concentrations the effectiveness of orientation and chemotactic migration decreases (112, 114). Cells observed in a visual chemotaxis assay system spread and flatten after the addition of the agonist, but begin to migrate after a few minutes, and soon about 80% of them has oriented and moves toward the highest concentration of the agonist (112). As they move up the gradient, they maintain a migrating polarized morphology, and ruffle only at their front (112, 84).
Chemotaxin-sensitivity, c.q. the dissociation constant of chemotaxin-binding, is adapted during agonist stimulation. Low amounts of chemotaxins increase the sensitivity to this agonist (115). Chemotaxin-concentrations which are high enough to elicit neutrophil activation decrease the sensibility to this agonist (46, 114-116), which might ensure ongoing detection of, and responsiveness to further increases in chemotaxin concentration on their path of migration (84).
In parallel, sensibility for most other chemotaxins increases (101, 115, 116) which might optimize neutrophil chemotaxis in presence of gradients of low amounts of other agonists. The latter adaptation has been referred to as neutrophil-priming (115, 117). Seen from this viewpoint, observations of specific desensibilisation for IL-8 and C5a of neutrophils isolated from pustules from patients suffering from relapsing bullous staphyloderma (118) are congruent with an *in vivo* role for C5a and IL-8 in chemotaxis.

B5. Neutrophil chemotaxis; roles of β_2 integrins

Additionally to the increase in avidity of the neutrophil β_2 integrins CD11a/CD18 and CD11b/CD18 (40-43), neutrophil agonist exposure leads to translocation of CD11b/CD18 from an intracellular pool to the cell surface (41, 119). During chemotaxis CD11b/CD18 is preferentially inserted at the leading edge of cell-motility (120).
Initial activation induced adhesion strengthening occurs, however, mainly by an activation of

pre-expressed CD11b/CD18 (40, 46), and lasts only a few minutes; only after a further increase in the stimulus the newly expressed CD11b/CD18 acquires an active form and mediates migration (46). Whilst *in vitro* migration of neutrophils adherent to protein coated glass or plastic as well as *in vivo* neutrophil accumulation at inflammatory sites can be blocked effectively by CD11b/CD18 specific antibodies (8, 46, 120b, 121, 122), trans-endothelial migration is reduced by both CD11a/CD18 and CD11b/CD18 specific antibodies (3, 123, 124).

The latter findings support the conclusion that CD11a/CD18 functions in locomotion of neutrophils, once its ICAM-ligands are available. Since there is no known rapidly mobilizable intra-cellular pool of CD11a/CD18 and neutrophil activation causes no increase in the amount of this integrin on the cell surface (41, 119), a role for CD11a/CD18 in mediating migration could be explained by temporally limited focal increases in avidity of CD11a/CD18. Spatially controlled avidity of CD11a/CD18 and probably also CD11b/CD18 could cooperate with spatial distribution of newly expressed CD11b/CD18, in converting local neutrophil agonist activation in locally increased binding strength, and thus into locomotion to the site with highest agonist-presence. Evidence for continuous activation and deactivation of CD11b/CD18 during adhesive interactions (125) and the finding that freezing of adhesion molecules in a state of high-avidity blocks eosinophil migration (126) supports such a hypothesis.

B6. Neutrophil chemotaxis; conclusions

Neutrophil chemotaxis as described above, can be interpreted as a balanced way of agonist stimulation, in which the neutrophil carefully regulates a setpoint beyond which agonists stimulation leads to increased expression of CD11b/CD18 and activation of CD11a/CD18 and CD11b/CD18. This setpoint is continuously adapted in a way that an chemotaxin-concentration exceeding the previous concentration leads to renewed activation. This principle has been described as temporal gradient sensing, and is thought to guide not only chemotaxis of neutrophils (127, 128) but also chemotaxis of bacteria (129). In neutrophils, but not in bacteria, temporal gradient sensing cooperates with a spatial gradient sensing mechanism (84, 127). The polar shape of migrating neutrophils seems to be correlated with focal expression and activation of CD11b/CD18 (46, 120), and therefore with focal adhesion-strengthening and directed migration. Seen from this view, the initial stretching-response seen rapidly after sudden massive agonist stimulation could result from 'over-stimulation' of this sensing mechanism of neutrophils, leading to generalized in stead of focally increased β_2 integrin avidity. The time needed to adapt the set point of this sensory mechanism could correlate with the duration of the initial stretching response during which locomotion is inhibited.

Rapid neutrophil respiratory burst is linked in time to the initial stretching response which precedes migration after sudden agonist exposure. Whether a causal principle exists which links respiratory burst activity to neutrophil stretching or to either subcortical F-actin distribution or generalized β_2 integrin activation, two phenomena which seem to be associated with neutrophil stretching, remains uncertain. The existence of such a link implicates a functional antagonism between neutrophil migration and neutrophil respiratory burst activity. Experi-

mental evidence is congruent with this antagonism. Cytochalacin B, a fungal metabolite which interferes with F-actin formation, interferes with migration (130) and highly increases and prolongs respiratory burst activity of activated neutrophils (87, 109, 115, 131, 132). The antagonism between neutrophil migration and neutrophil respiratory burst activity, e.q. the association between neutrophil respiratory burst activity, generalized β_2 integrin activation, and generalized subcortical F-actin distribution, is further emphasized in the following chapters on prolonged respiratory burst activity.

B7. Neutrophil respiratory burst activity

Since 1961 it has been observed that during phagocytosis of bacteria, zymosan, and particles such as latex beads, neutrophil oxygen consumption is increased, and oxygen radical release in phagosomes can be measured (133-135). About 15 years later, the potency of chemotaxins such as FMLP and C5a to induce a phagocytosis independent respiratory burst became evident (109, 136-138). Chemotaxin induced respiratory burst activity needed relatively high concentrations of chemotaxins (109), was lower (109, 136, 138) and lasted shorter (109, 136) than phagocytosis induced oxygen radical production.

The last ten years, growing attention for neutrophil respiratory burst activity induced by a mechanism which seems to be a combination of phagocytosis induced respiratory burst activity and respiratory burst activity induced by chemotaxins and other soluble neutrophil agonists has risen. In a series of publications, Nathan et al. (139-143), Fehr et al. (106, 114, 144-146), and Kownatzki and Kapp et al. (147-152) showed that neutrophils adherent to artificial surfaces ('frustrated phagocytosis' model) respond different to addition of high amounts of chemotaxins and other agonists in comparison to non-adherent neutrophils. Adherent neutrophils show a highly prolonged and increased respiratory burst, starting after a 'lag time' of 15 to 60 minutes, incited not only by chemotaxins, but also by a group of other agonists which lack chemotactic activity and fail to induce any respiratory burst activity by suspended neutrophils. The following chapters and part C further specificate the latter form of respiratory burst induction, and present evidence for its (patho)physiological relevance.

B8. Slow neutrophil responses; substrate induced neutrophil spreading and prolonged respiratory burst activity

In the previous chapters, the response on activation of neutrophils in contact with a substrate for adherence, has been described as a transient stretching response followed by a polar shape and migration. Substrate contacting neutrophils can, however, also show another response, during whitch initial stretching continues, resulting in highly flattened neutrophils which do not migrate. This response can be induced *in vitro* by placing resting neutrophils on uncoated polystyrene or glass surfaces as a substrate for adherence (139, 153). In parallel to extensive neutrophil spreading and flattening, F-actin localizes in small foci on the adherent surface (154). Even in absence of soluble agonists, neutrophil spreading on uncoated polystyrenetriggers strong and ongoing respiratory burst activity (104, 139, 141, 153). The

increase in F-actin content (154), and initiation of respiratory burst (104, 139) start rapidly, but develop more slowly than during the rapid responses on stimulation with chemotaxins, and persist much longer. Polystyrene induced neutrophil respiratory burst can be easily reduced by altering its surface characteristics. Lowering the negative surface charge (141), surface-coating with serum (141, 139), with specific proteins (104, 139-141), or with a monolayer of cells (139) suppresses respiratory burst activity completely, or delays the time of onset.

Activation of such substrate contacting neutrophils with very high amounts of FMLP or C5a induces increased and highly prolonged respiratory burst in comparison to activation of suspended neutrophils (104, 106, 139, 143, 147). Moreover, the neutrophil agonists TNF, GM-CSF and LPS, which fail to induce rapid respiratory burst activity by suspended neutrophils, induce prolonged massive oxygen radical release when added neutrophils in contact with adequate surfaces (75, 106, 139, 140, 141, 148, 149, 151, 155, 156).

Rapid neutrophil respiratory burst activity, as defined previously, begins within 1 minute and lasts up to 30 minutes. Prolonged respiratory burst activity by neutrophils in contact with uncoated polystyrene or glass also begins rapidly, but lasts for hours (104, 139, 141, 153). Prolonged respiratory burst activity by neutrophils in contact with coated polystyrene or glass in response to adequate agonists activation (for details chapter C1) is characterized by a lag period of 15 to 60 minutes after which ongoing respiratory burst can be measured (75, 104, 139, 140). These different manifestations of neutrophil respiratory burst activity can be linked to actin-organization and adhesion molecules.

B9. Neutrophil spreading and prolonged respiratory burst; role of F-actin

Both neutrophil flattening (154, 157) and prolonged respiratory burst (139, 142, 153, 155) can be prevented by addition of cytochalasin B. Cytochalasin B, which interferes with F-actin formation, prevents the switch from initial adherence to migration (158) and prolongs rapid respiratory burst activity correlated with initial adhesion (87, 109, 115, 131, 132).

Cytochalasin B also interferes with cell spreading (154, 158), and inhibits initiation of prolonged massive respiratory burst activity correlating with cell spreading (139, 142, 153 ,155). This double action of cytochalasin B, increasing rapid respiratory burst activity and inhibiting prolonged respiratory burst activity, has not been explained so far. A direct relation between the cytoskeleton and the enzymes responsible for respiratory burst activity (159) might be responsible for these influences of cytochalasin B.

Another attractive idea is that cytochalasin B acts on respiratory burst activity indirectly, by inhibiting alterations in the shape and behaviour of neutrophils. Both the inhibition of migration and the inhibition of ongoing flattening might interfere with substrate-interaction dependent CD11b/CD18 functions linked to respectively stopping and beginning of respiratory burst activity. This option would implicate a role of F-actin in facilitating specific CD11b/CD18 mediated adhesive interactions.

B10. Neutrophil spreading and prolonged respiratory burst; role of β_2 integrins

Extensive neutrophil spreading and flattening is linked to prolonged respiratory burst activity. Both neutrophil flattening and prolonged respiratory burst activity are dependent on CD11b/CD18 function (75, 104, 140, 142, 160). In specific *in vitro* conditions, CD11b/CD18 function can be inhibited whilst neutrophil attachment or flattening can be facilitated by CD11a/CD18-ICAM-1 interactions, selectin interactions, or lectin-mediated binding. These conditions however, do not allow prolonged respiratory burst activity in response to the neutrophil agonist TNF (75, see chapter C1), indicating that CD11b/CD18 does not just facilitate neutrophil flattening, but has a more direct role in prolonged neutrophil respiratory burst activity. Moreover, a number of polystyrene coatings, such as stearic acid, collagen, albumin inhibit prolonged respiratory burst activity without (completely) inhibiting CD11b/CD18 mediated attachment (104, 107, 139, 140).

β_2 integrins seem to have signal transducing capacities, since changes in cytosolic free Ca^{++} and c-CAMP, and changes in cellular behaviour can be initiated by β_2 integrin substrate contact and by crosslinking of α and β chains by specific antibodies (142, 161-165). A CD11b/CD18 submitted activation signal, depending on a specific state of CD11b/CD18, might thus be a prerequisite for prolonged neutrophil respiratory burst activity.

Whilst neutrophil chemotaxis seems to be linked to focal increases in CD11b/CD18 avidity, a generalized increase in CD11b/CD18 avidity seems to be correlated with both rapid and prolonged neutrophil respiratory burst activity. Direct evidence for a generalized highly avid state of surface CD11b/CD18 (and probably also CD11a/CD18) during ongoing respiratory burst activity is lacking until now.

The observation that initial CD11b/CD18 mediated neutrophil attachment to coated polystyrene requires static conditions, whilst after a short period of time, neutrophils become highly flattened and neutrophil attachment acquires resistance to extreme shear stresses (37, 49, 142) clearly reflects a switch to generalized highly avid CD11b/CD18 mediated substrate interactions. Part of the mechanism by which this switch is regulated has recently been demonstrated by Nathan and all. and is presented in the next chapter.

B11. Neutrophil spreading and prolonged respiratory burst; role of CD43

Neutrophil adhesion is the result of a complex interaction of adhesive and anti-adhesive factors. Besides specific receptor-ligand interactions, hydrodynamic forces, and the arrangement of the cytoskeleton, specifically regulated electrostatic forces participate in determining neutrophil adhesion. Abrahamson suggested more than 60 years ago that a decrease in negative surface charge might facilitate the adhesion of neutrophils to the negatively charged endothelial cell surface during the inflammatory response (166). Resting neutrophils and other leukocytes express a long rigid sialoprotein with marked negative charge, known as CD43 (sialophorin, leukosialin). Parallel to neutrophil activation, and probably resulting from the release of proteases such as elastase and sialidase from activated neutrophils, adherent neutrophils can shed CD43 from their surface (142, 167-169). CD43 prevents neutrophil flattening, whilst

leaving initial adherence unaffected (143). The mechanism for this effect appears to be linked to specific characteristics of CD43. The extended extracellular domain of CD43, which protrudes 45nm from the plasma membrane on rat T-cells (170), farther than expected for any molecule on the surface of these cells, the rigidity, and the high negative charge (Pi=4.1) (171), make it plausible that for integrins or other adhesion molecules to mediate flattening of neutrophils, neutrophils must first shed, desialylate, and/or redistribute CD43 away from points of contact (143). CD43 thus acts as an 'anti-adhesive' molecule, which appears to prevent prolonged neutrophil respiratory burst activity and favor (chemotactical) migration for as long as its normal expression has not been affected.

B12. Neutrophil spreading and prolonged respiratory burst; role of albumin

Human serum albumin binds to surface CD43, and suppresses both agonist and protease induced CD43 shedding (143). This mechanism is likely to explain previous observations of the capacity of human serum albumin to prevent agonist induced prolonged respiratory burst activity (140, 143), to reduce F-actin formation correlated with neutrophil flattening (154), and to increase neutrophil chemotaxis (172-174). Thus, the impact of albumin on neutrophils is likely to be biphasic during the evolution of an inflammatory response. In healthy organisms, the high intravascular albumin concentrations may favor neutrophil-extravasation, and the low extravascular albumin concentrations may facilitate neutrophil retention in the extravascular tissues, where they may be relatively free to spread, degranulate, and undergo a respiratory burst. Reduction of the trans-vascular gradient of albumin in later stages of inflammation may have the opposite effects (143).

B13. Slow neutrophil responses; conclusions

Agonist activation of resting neutrophils induces a rapid initial phase, characterized by generalized subcortical F-actin deposition, generalized CD11b/CD18 activation, and, after adequate agonist exposure, a transient respiratory burst and protease-release. This initial adherence-independent phase probably occurs in response to any neutrophil agonist of sufficient strength. Neutrophils adapt rapidly by decreasing their sensibility to the initial agonist, and a switch to a polar shape with focal CD11b/CD18 activation and F-actin deposition can be observed. This switch can, however, be prevented by extensive neutrophil spreading mediated by CD11b/CD18 and by CD43 shedding. Both CD11b/CD18 mediated binding and CD43 shedding are highly regulated. Extreme conditions, such as neutrophil activation by the croton oil derived protein kinase C activating carcinogen PMA, triggers a prolonged respiratory burst independent from CD11b/CD18 mediated substrate contact (107, 142, 147) and independent from albumin presence (143). Also, uncoated polystyrene surfaces trigger a prolonged respiratory burst independent from agonist presence (104, 139, 141, 153). In more physiological circumstances agonist conditions, substrate conditions and other environmental conditions such as albumin presence cooperate in determining whether the initial neutrophil response is followed either by a polar shape and migration or by increased flattening and prolonged neutrophil respiratory burst activity.

Chapter C

DIFFERENCES BETWEEN NEUTROPHIL AGONISTS; A SPECIFIC ROLE FOR TNF?

C1. Chemotaxis-inducing and respiratory burst-inducing agonists; *in vitro* evidence

The response of neutrophils to agonists can be divided in a general initial response, and in specific prolonged responses. Reorganization of the neutrophil surface receptor make up seems a central feature in the initial response of neutrophils to agonist activation. This is indicated by the increase in CD11b/CD18 expression and by neutrophil priming, the latter being partly related to altered receptor expression (175, 176), found as common responses to the presence of the agonists indicated in Table 1.
However, the sensitivity of neutrophils to low molecular weight agonists such as C5a, FMLP, PAF, leukotriene B_4 and LPS tends to be lower in comparison to protein-agonists TNF-α, TNF-β and GM-CSF. Shedding of L-selectin from the neutrophil surface is another common response, showing similar kinetics as CD11b/CD18 upregulation (65).

A clear divergence between different agonists is seen in their capacity to induce chemotaxis, and in the concentration required to induce respiratory burst activity *(table 1)*. On the basis of this divergence, neutrophil agonists can be divided into two groups. The first group, to which TNF-α, TNF-β, GM-CSF and LPS belong, elicit a substrate-dependent prolonged respiratory burst as a slow response to concentrations which also elicit CD11b/CD18 upregulation and priming. TNF, GM-CSF and LPS are not chemotactically active, but inhibit migration towards chemotaxins (144, 148, 177-181).
The second group, to which IL-8, FMLP, C5a, PAF and leukotriene B_4 belong, elicit chemotaxis as a slow response to concentrations which also elicit CD11b/CD18 upregulation and priming. Only at higher concentrations, these agents elicit a rapid respiratory burst (mainly in presence of cytochalasins) or a prolonged respiratory burst (in contact with appropriate surfaces). Respiratory burst activity occurs at 10 to 100 times the concentrations required for chemotaxis or CD11b/CD18 upregulation for IL-8, C5a and FMLP, and at more than 100 times higher concentrations of PAF and leukotriene B_4 *(table 1)*.

It seems legitimate to conclude that the role of IL-8, C5a, and FMLP in neutrophil behaviour *in vivo* is limited to chemotaxis, and that term 'chemotaxins' applies very well to these agonists. *In vivo* situations in which the exposure of neutrophils to the latter agonists is abrupt enough to avoid adaptation, and high enough to trigger a respiratory burst, will not easily occur. In parallel, neutrophil priming by IL-8, C5a and FMLP can have a function in chemotaxis, in which priming optimizes migration towards low amounts of other chemotaxins. Neu-

trophil priming by these chemotaxins is unlikely to cooperate in neutrophil respiratory burst activity. Priming does increases the rate of oxygen radical production but hardly decreases the threshold concentration needed to induce a respiratory burst by chemotaxins (87, 117, 182). Also, neutrophil priming by IL-8, C5a and FMLP seems to fail in increasing respiratory burst in response to the first group of agonists. TNF-induced respiratory burst activity is not increased by either endothelial cell released or added IL-8 (76), by C5a (own unpublished observation) or by FMLP (148). Moreover, previous activation by such agonists decreases the sensitivity of neutrophils for TNF, by inducing rapid shedding of the TNF-receptor (156, 183). Clear priming effects of FMLP, C5a or IL-8 on neutrophil toxicity elicited by GM-CSF or LPS have not been described.

Table I.

Comparison of neutrophil activation by some potential physiological soluble agonists.

Agent	EC_{50} (*M*)					References
	chemotaxis	CD11b/CD18 upregulation	priming	rapid respiratory burst	prolonged respiratory burst	
TNF-α	absent	10^{-11}-10^{-10}	10^{-11}-10^{-10}	absent	10^{-11}-10^{-10}	75, 139, 148, 151, 178, 179, 180, 184, 185
TNF-β	absent	not specified	not specified	absent	$2\text{-}7\times10^{-10}$	139, 148, 151, 186
GM-CSF	absent	5×10^{-12}-10^{-10}	10^{-11}-10^{-10}	absent	4×10^{-11}-10^{-10}	103, 141, 149, 187-191
LPS	absent	1-10ng/ml	2-50ng/ml	absent	10-50ng/ml	110, 139, 144, 145, 192-195
IL-8	5×10^{-11}-10^{-9}	10^{-9}	10^{-9}	10^{-9}-2×10^{-8}	10^{-7}	59, 65, 116, 182, 196-201
FMLP	10^{-11}-10^{-9}	$1\text{-}7\times10^{-9}$	4×10^{-7}	4×10^{-8}-4×10^{-6}	10^{-8}-3×10^{-7}	39, 65, 104, 106, 109, 152, 188, 202-204
C5a	2×10^{-10}	4×10^{-10}-10^{-9}	10^{-9}	10^{-8}	$1\text{-}3\times10^{-8}$	65, 106, 152, 175, 197, 203, 205, 206
PAF	10^{-10}-10^{-9}	10^{-11}-10^{-9}	10^{-12}-10^{-7}	10^{-8}-5×10^{-8}	10^{-6}-10^{-5}	65, 206-215
Leukotriene B_4	10^{-9}-10^{-8}	10^{-10}-10^{-9}	10^{-8}	10^{-6}	unknown	65, 204, 206, 214, 216-221

C2. Chemotaxis-inducing and respiratory burst-inducing agonists; *in vivo* evidence

Evidence for a role of FMLP, C5a and IL-8 in chemotaxis, and for a role of TNF, GM-CSF, and LPS in neutrophil toxicity also arises from *in vivo* experiments. Systemic agonist administration leads to rapid neutropenia, resulting from neutrophil sequestration in the lungs and other organs. FMLP (95, 100, 222), C5a (95, 99, 100, 223) and IL-8 (97, 98) induced neutropenia is restored within less than one hour, without resulting in clear tissue or organ damage. In contrast, TNF (181, 224-226), GM-CSF (190, 227) and LPS (95, 96, 222, 224, 226) induced neutropenia holds on for at least two hours, and can result in organ injury (222, 225,

226, 228). Whilst chemotaxin-induced neutropenia results solely from an F-actin dependent increase in neutrophil stiffness (89, 90, 93), for LPS-induced neutropenia and involvement of CD11b/CD18 dependent anchoring of neutrophils in neutropenia at later time-points, has been demonstrated (94).

C3. A specific role for the lipid agonists PAF and leukotriene B_4?

In comparison to the other neutrophil agonists, PAF and leukotriene B_4 are weak inducers of neutrophil respiratory burstactivity; only after addition of relatively high concentrations of PAF (208, 210, 211) or leukotriene B_4 (204, 216, 218, 229,230) a low and short-lived respiratory burst can be measured. In sharp contrast with the µM concentrations of PAF needed to elicit respiratory burst activity of resting neutrophils, 10^{-12}M of PAFwas shown to increase TNF induced neutrophil respiratory burst effectively (209).

Co-production of PAF and leukotriene B_4, by neutrophils itself or by surrounding cells, occurs in many instances during neutrophil mediated inflammatory processes (56, 56b, 56c, 213, 231-234). Evidence for involvement of endogenous PAF (209, 232, 235) and leukotriene B_4 (204) in neutrophil respiratory burst activity, and for amplification of the respiratory burst by endothelial cell associated PAF (76, 233, 236) suggest that these lipid-mediators might have a regulatory role in many, if not all, pathophysiological situations associated with activation of the neutrophils respiratory burst.

C4. Neutrophil-agonists; conclusions

Focussing on prolonged neutrophil responses, a hypothetical devision of common soluble neutrophil agonists into three groups is made.

TNF, GM-CSF and LPS induce prolonged respiratory burst activity but not chemotaxis. IL-8, FMLP and C5a induce chemotaxis at low, and prolonged respiratory burst activity at high concentrations. PAF and leukotriene B_4 induce chemotaxis and prime neutrophils for a prolonged respiratory burst in response to other agonist, but are relatively incapable of triggering a respiratory burst by resting neutrophils. The kinetics of the F-actin responses to the agonists support this division.

The first group of agents induce an increase in F-actin content which remains high for a long period of time (87, 94, 237). The second group of agents induce only a temporal increase in F-actin content, although the decline in F-actin content can be counteracted by increasing the agonist-concentration (83, 85, 88, 229). After F-actin formation induced by the third group of agents, a sharp decline, followed by ongoing oscillations in the F-actin content is found (88, 229, 238).

A hypothetical functional characterization of the different agonist-groups can be postulated from these data. IL-8, bacterial formylated peptides (represented by FMLP), and C5a could have a main role in directing neutrophils to the inflammatory site. This function involves focally increased F-actin content.

Once neutrophils arrive in a zone with sufficient TNF, LPS or GM-CSF (or chemotaxin-concentrations too high to adapt to), the focal F-actin concentration is replaced by a general

increase in F-actin, migration is reduced, and facilitated by CD43 shedding and highly avid CD11b/CD18 mediated substrate contact, the neutrophil flattens and becomes functionally active by releasing high amounts of proteases and reactive oxygen metabolites. Locally produced leukotriene B_4 but especially PAF could have a co-regulatory role, enhancing both chemotaxis and prolonged respiratory burst activity.

C5. TNF as a specialized regulator of neutrophil toxicity

There is presumably no other cytokine, which has such a wide range of activities on so many cell types as TNF (239). TNF is released by hemopoietic cells, renal epithelial cells (240, 241), and by some tumor cells (242, 243). Two forms of TNF, TNF-α (cachectin) released mainly by mononuclear phagocytes, and TNF-β (lymphotoxin) released by lymphocytes, have been identified, which show about 35% sequence similarity (244, 245), and act via the same receptors (reviewed in 246). Although circulating free TNF can be found temporarily after acute and severe inflammatory assaults, and a number of systemic inflammatory responses are mediated by TNF, TNF is thought to function in host defence mainly according to a paracrine model in which TNF is produced and acts locally (reviewed in 247). TNF has a number of characteristics which fit very well with a key role in regulating neutrophil toxicity:

1. TNF elicits endothelial cell mediated neutrophil rolling. Together with IL-1, which lacks the capacity to induce prolonged neutrophil respiratory burst activity (139), TNF is the most powerful known physiological inducer of E-selectin expression by the endothelial cell vessel lining (24, 248, 249). An *in vivo* role for TNF in mediating E-selectin expression in situations of LPS induces septic shock has been demonstrated (250).
2. TNF induces endothelial cell mediated neutrophil attachment and transmigration. Parallel to E-selectin expression, TNF induces endothelial cell associated PAF (56, 56c), endothelial cell IL-8 release (57, 241) and increased expression of endothelial cell ICAM-1 expression (249, 251). PAF, IL-8 and ICAM-1 mediate neutrophil attachment and transmigration (3, 56, 62, 123).
3. TNF directly induces prolonged neutrophil respiratory burst activity and can be indirectly responsible for neutrophil chemotaxis. TNF has a direct effect on neutrophils, leading to inhibition of chemotactic migration (148, 178, 186, 179, 180), neutrophil spreading (142), a prolonged respiratory burst (75, 139, 140, 148, 151, 155, 156) and protease release (102, 156, 252, 253). This response is incited at TNF-concentrations of 10^{-11}M and more.

Indirectly, via induction of IL-8 release by other tissue cells such as fibroblasts (254, 255), renal epithelial cells (241, 256) and hepatocytes (257), a TNF gradient can induce an IL-8 gradient, which has strong chemotactic properties.

In vitro medium concentrations of 10^{-10}M IL-8 are measured within hours after incubation of renal epithelial cells with only 6×10^{-13}M TNF (241). TNF concentrations which are 10 to 100 times belowconcentration required to induce inhibition of chemotaxis thus might induce neutrophil chemotaxis indirectly by enhancing IL-8 release. Only once neutrophils have migrated to a region with local TNF-concentrations higher than 10^{-11}M, accumulation of neutrophils,

and subsequent triggering of neutrophil toxicity might occur. *In vivo* evidence on these statements is limited, since adequate and selective inhibition of local TNF-production or TNF-bioactivity remains difficult. After local TNF-administration, however, clear neutrophil accumulation and local tissue damage are found (258-260).

4. Activation of neutrophil elastase release limits TNF-presence. TNF induces the release of neutrophil secondary granule constituents (102, 156, 252, 253). Elastase and catchepsin G, two major proteases stored in secondary granules, can inactivate TNF by proteolytic cleavage (261, 262, 263). Furthermore, proteolitic cleavage by elastase induces shedding of the neutrophil TNF-receptor, and therefore decreases the sensitivity of neutrophils to TNF (264). Thus, a feed-back mechanism can be postulated, which limits TNF-induced neutrophil accumulation and neutrophil toxicity, via inactivation of TNF and neutrophil TNF-receptors once a certain concentration of elastase (and a breakdown of the anti-protease shield by reactive oxygen species) is achieved.

5. Systemic neutrophil activation limits the bioactivity of systemic TNF as well as neutrophil sensitivity to TNF. Neutrophil activation leads to shedding of the TNF-receptor of neutrophils (156, 183). Shedded TNF-receptors are capable of inactivating TNF (265, 266), whilst neutrophils which have shed their TNF-receptors show decreased responses to TNF (156, 267). Tissue injury as a result of neutrophil sequestration in the lungs and liver following severe trauma will, for example, be counteracted by this decrease in sensibility for local TNF. Additionally, systemic presence of neutrophil agonists will result in high concentrations of circulating TNF receptors, which can buffer TNF leaking into the circulation, and therefore reduce the potentially lethal responses to systemic TNF (183).

6. TNF specifically cooperates in neutrophil mediated inactivation of foreign structures. Neutrophilic granulocytes are specialized in digesting foreign material. Respiratory burst activity and degranulation are essential in this process, and are activated either during phagocytosis or after intense spreading (see chapter B7 and B8). Both phagocytosis and intense spreading are thought to result directly from increased adhesive interactions; the only principle difference is the size of the particle to which the neutrophils attaches (268).

Regulation of binding, phagocytosis and respiratory burst activity are closely linked processes. Direct binding of foreign structures can occur via CD11b/CD18, which has affinity for the capsule-components of gram negative bacteria and yeast, specific parasite-components, and plastic surfaces (9, 10, 269, 270).

CD11b/CD18 mediated interactions with yeast particles directly incite a process of progressive attachment, phagocytosis, and prolonged respiratory burst activity (104, 153, 269, 271). TNF can cooperate in this cascade by strongly enhancing phagocytosis of particles and by increasing respiratory burst activity (272-275).

The induction-profile of TNF-release matches very well with a role for TNF in supporting particle-digestion by neutrophils. Attachment of mononuclear phagocytes to plastic (276) or phagocytosis of yeast particles (277, 278) specifically induces TNF gene transcription or release. Furthermore, surface-bound immuno-globulins (279, 280) and surface-bound complement (277) are strong and specific triggers for TNF release by mononuclear phagocytes.

Thus, the specific production of TNF in response to foreign structures, and the capacity of TNF to enhance of their digestion by neutrophils, might imply a crucial role for TNF in coping with foreign substances by the immune-system.

C6. GM-CSF, a potential regulator of neutrophil toxicity?

GM-CSF is a hemopoietic growth factor with substantial effects on the proliferation of myeloid leukocytes. Its *in vitro* effects are, however, not confined to myelopoieses, but identify GM-CSF as a powerful regulator of myeloid leukocyte functions (reviewed in 281 and 282). GM-CSF lacks, however, most effects of TNF on non-myeloid cells. Induction of endothelial E-selectin, ICAM-1, PAF and IL-8, for example, has not been reported. T-cells, macrophages, mast-cells, endothelial cells and fibroblasts can be triggered to release GM-CSF, and GM-CSF has pleiotropic connections within the cytokine-network. Close connections with TNF exist: TNF enhances transcription of GM-CSF (283-285) and enhances GM-CSF induced effects (286) whilst GM-CSF also induces production of TNF (286-289). GM-CSF is thought to be produced and act locally, since detectable levels of GM-CSF in the circulation have not been found (281, 282). *In vivo* evidence, demonstrating local presence or involvement of GM-CSF in (patho)physiological processes, has, however, been very limited (290, 291). Whether GM-CSF plays an actual role in homeostatic maintenance of myelopoiesis and in host defence thus remains to be answered.

C7. LPS, a potential regulator of neutrophil toxicity?

Bacterial lipopolysaccharides (LPS or endotoxin), the principal lipid component of the outer leaflet of the envelope of gram-negative bacteria, is recognized by, and elicits strong responses in essentially all multicellular organisms. Recognition of LPS in humans depends on complexation of LPS with specific serum proteins and subsequent binding to CD14 (292-295). Mononuclear phagocytes express high amounts of CD14 (about 50.000 molecules/cell) (296) and respond to low LPS-concentrations (EC_{50}10~100pg/ml) (292, 295, 297). Neutrophils express smaller amounts of CD14 (195, 298) and respond to higher LPS-concentrations (EC_{50}1-50ng/ml) (Table 1). Tissue cells such as endothelial cells do not clearly express CD14 (299, 300), and respond only to very high LPS-concentrations (EC_{50} up to 1μg/ml). However, we could show that this response is mediated by CD14 (251), which might originate from mononuclear phagocytes (299-303). Mononuclear phagocytes thus seem to be specialized in detecting small amounts of LPS, and in responding in an earlier stage during LPS-challenge. TNF release by these cells starts within one hour after LPS-exposure, and TNF mediates many of the effects seen after experimental LPS-administration (250, 304-306). The direct effects of LPS on neutrophils (inhibition of chemotaxis, prolonged F-actin formation, stretching, degranulation and induction of a prolonged respiratory burst (144, 94, 139, 145), as well as the indirect effects (induction E-selectin, ICAM-1, and IL-8 (24, 248, 251) which mediate respectively neutrophil rolling, neutrophil attachment, neutrophil transmigration and chemotaxis), are similar to the effects of TNF. The LPS concentrations needed to induce these effects are, however, $10^3$to 10^4 times higher than concentrations of LPS needed to induceTNF

release by monocytes, and even at sufficient concentrations, the effects of LPS on neutrophils (139) and on endothelial cells (24, 251) generally remains lower than the effects of TNF. The direct response to LPS thus seems to be delegated to a selective cell population, whilst other cells use a signal of thisspecialized cell to respond indirectly to LPS (303). Furthermore, the effectiveness of experimental administration of TNF-specific antibodies in counteracting systemic LPS-responses (304, 305), *in vivo* endothelial cell responses (250) and neutrophil responses (306) to LPS or bacteremia, argues for the importance of a TNF-mediated indirect effect of LPS. Whether the direct pathway, activation of cells such as neutrophils and endothelial cells by LPS, has still physiological relevance, remains to be determined. Specific situations might exist during which the indirect pathway is nonfunctional. In a disease such as paroxysmal nocturnal hemoglobinuria in which cell-associated CD14 is lacking, in the extravascular space, or after LPS-desensitization of myeloid cells by prolonged contact with LPS, the direct pathway of endothelial cell activation might be crucial for an inflammatory response to LPS (303).

C8. TNF as a specialized regulator of neutrophil toxicity; conclusions

The control of neutrophil toxicity is a major challenge in host defense. TNF has unique characteristics in regulating this neutrophil function. Specific local release by mononuclear phagocytes, several levels of control of bioactivity of and sensibility for TNF, and the potential to induce, partly indirect, neutrophil rolling, attachment, transendothelial migration, chemotaxis, and activation of neutrophil toxicity, give TNF a potential key-position in the control of neutrophil toxicity. Whether the role of TNF is indeed crucial in the regulation of neutrophil toxicity, remains a major question.

Evolution might have favored the appearance of a redundancy of inflammatory mediators with overlapping effects, thus minimizing the risk for perturbation of intercellular communication during crucial immune-responses. In vivo models in which TNF-mediated communication-signals are selectively and completely eliminated should provide part of the answer to this question. Recent techniques of raising TNF-receptor gene knock-out mice (307) might achieve this goal. In mice lacking the 55 kD TNF receptor only a increased sensitivity to infection with intracellular-living bacteria such as Listeria was observed (307). This finding could implicate that most functions of TNF are indeed relatively easily by-passed by other, maybe as yet undiscovered, mediators. Whatever the answer is, investigations on TNF have a unique place in the history of unraveling the answers host defence has developed in dealing with microbial challenge.

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Chapter 2

INTRODUCTION TO AND DISCUSSION OF THE EXPERIMENTS

The studies presented in this thesis are part of the rapidly evoluating fields in immunology of adhesion-molecules and cytokines. During the realization of the experimental work presented in this thesis, both knowledge and technical possibilities increased rapidly, and the questions which were asked and answered in the individual manuscripts represent the status quo of the moment at which they were written. The central theme of this thesis is the regulation of neutrophil-endothelial cell interaction during inflammation.
The experimental work presented in this thesis can be divided into three parts. Chapter 3 focusses on TNF. Chapter 4 focusses on E-selectin. In chapter 5, the involvement TNF and E-selectin in neutrophil endothelial cell interaction is investigated.

Chapter 3

In chapter 3 the conditions which lead to the release of TNF and related cytokines such as IL-6 and IL-8 were investigated. TNF is known to be a main mediator of the pathophysiological changes during gram negative sepsis, released mainly by mononuclear phagocytes (reviewed in 1). Lipopolysaccharide (LPS) from the outer leaflet of the envelope gram-negative bacteria, also called endotoxin, is the 'classical' agent responsible for TNF-release by mononuclear phagocytes.
Since TNF can be held responsible for mortality associated with experimental endotoxin induced sepsis syndrome (1), it seemed important to identify the mechanisms which determine whether TNF-release contributes to a controlled host-protective inflammatory response, or leads to a toxic overshoot-reaction with potentially lethal outcome.

In the first paper of this chapter, it was shown that TNF is not only associated with gram negative sepsis, but can also be partly responsible for the sepsis syndrome induced by zymosan (purified ghost cells of bakers yeast). In our model, the trigger for massive TNF-release appeared not to be restricted to one specific agent, such as endotoxin, but was the result of a combination of triggers: the zymosan-particles themselves, particle bound serum components and activated soluble serum components.
Further evidence that TNF-release is rather a highly regulated multifactorial event than an isolated response of a single cell-population to one toxic agent, is presented in chapter 3.2 and 3.3.
Renal epithelial cells share some properties such as phagocytosis and oxygen radical release with mononuclear phagocytes. In chapter 3.2 we demonstrated that cultured explanted human renal epithelial cells responded to cytokines and endotoxin by releasing TNF, IL-6 and IL-8.

These findings emphasize that renal epithelial cells are not just target cells during inflammatory challenge, but can participate directly in the regulation of ongoing inflammation.
As indicated by the name 'endotoxin', lipopolysaccharides have long been considered as host threatening microbial poisons. Serum components were thought to play an host-protecting role by 'detoxificating' LPS (2, 3). More recent investigations pointed out that the immunogenicity of LPS is influenced by two homologous proteins: LBP, an acute phase reactant released by the liver, which highly increases LPS binding to CD14, and BPI, which is produced by activated neutrophils and is capable of inactivating LPS.
Experiments on the interactions of these proteins, performed in close collaboration with Mieke Dentener in our laboratory, demonstrated that BPI can prevent association of LPS with LBP and thus prevent LPS-induced cytokine-release by mononuclear phagocytes (paper 3.3). BPI thus represents a physiological regulator of LPS-toxicity, which could be important in preventing LPS-induced septic shock-like syndromes. Thus, the recognition of endotoxin by cells of the immune-system represents another level at which the TNF-response is rather balanced by specific other host mediators, than an isolated reaction to a 'toxic' microbial agent.

Chapter 4

E-selectin is an endothelial cell adhesion molecule for neutrophils and other cells which is temporarily expressed after endothelial cell activation with TNF and other pro-inflammatory agents, and has a main role in the recruitment of neutrophils at sites of inflammation. Several aspects of this novel adhesion molecule were investigated in close collaboration with Jet Leeuwenberg, using two monoclonal antibodies produced at our laboratory.

We were interested in possible functional effects of E-selectin interaction with its ligand on neutrophils. Whilst our investigations on the effects of E-selectin-ligand binding on the behavior of neutrophils and endothelial cells were negative*, we observed that E-selectin is not stably expressed by endothelial cells but is internalized after a short stay on the cell surface. E-selectin binds soluble L-selectin, which is released from the neutrophil surface after activation (4, 5) and other soluble sialyl Lewis X moieties containing proteins, which are found in the circulation of some adenocarcinoma-patients (6, 7) and of patients with an acute generalized inflammation (8). Both such proteins can reduce neutrophil adherence to activated endothelium (8, 9). Furthermore, E-selectin contains six repetitive regions with sequence homology to known complement regulatory proteins (10). As suggested by Johnston et al. (11), these regions might bind C3b or C4b to facilitate clearance of circulating immune-complexes. E-selectin internalization, as described in paper 4.1, could have a function in decontaminating the circulation during severe immunological challenge. Furthermore E-selectin internalization could be involved in the downregulating of E-selectin expression, which

* Neutrophil interaction with E-selectin fails to trigger respiratory burst activity (chapter 2.2), but induces chemotaxin-like neutrophil activation as was demonstrated later in a study of S. K. Lo et al. (4).

occurs some 4 to 8 hours after the initiation of E-selectin expression. The mechanism of the induction and downregulation of E-selectin expression was further investigated in the chapters 4.2, 4.3 and 4.4. Three mechanisms are described in these chapters, which co-regulate E-selectin expression. IFN-γ, which fails to induce E-selectin expression by itself, increases E-selectin and other endothelial cell responses to TNF, IL-1ß and LPS (chapter 4.2), as was confirmed by parallel investigations of Doukas and Pober (12). Presence of so far unidentified heat labile serum-components during endothelial cell activation appeared to be essential in facilitating full E-selectin expression (chapter 4.3).
The kinetics of E-selectin expression were, however, not affected by presence of IFN-γ or serum. Serum-presence appeared particularly important for full endothelial cell responses to LPS. Involvement of serum-components and of the cell surface protein CD14 in monocyte-activation by LPS had just been elucidated by S. D. Wright et al (13, 14).
Data which show that CD14 molecules are crucially involved in LPS-activation of endothelial cells are presented in chapter 4.4, and are supported by simultaneous reports by other groups (15-17).

Chapter 5

As depicted in chapter 3 and 4, TNF is part of a highly regulated network of mediators which modify both the release and the effects of TNF. Part of the influence of TNF on neutrophil-endothelial cell interaction is induced indirectly, by the induction of E-selectin on endothelial cells, by effects on other adhesion molecules involved in endothelial-neutrophil interaction such as ICAM-1 and CD11b/CD18, by effects on other inflammatory mediators such as IL-8, which are released by endothelial cells in response to TNF, and by PAF expressed by endothelium in response to TNF-activated neutrophils (chapter 1).
In chapter 5, the involvement of TNF, and of TNF-regulated adhesion-molecules and inflammatory mediators in neutrophil endothelial cell interaction is investigated, focussing on neutrophil mediated endothelial cell damage. Additionally, TNF-induced neutrophil activation was compared with neutrophil activation by an neutrophil binding elastase reactive monoclonal antibody, used as a model for the influence on neutrophils of anti-neutrophil cytoplasmic antibodies which are commonly found in systemic vasculitides such as Wegener's granulomatosis (chapter 5.2).
E-selectin appeared not to be involved directly in neutrophil mediated endothelial cell damage, but to have a specific role in neutrophil adhesion (chapter 5.3). This in contrast to the neutrophil adhesion molecule CD11b/CD18 which, besides mediating adhesion, has an essential role in facilitating neutrophil respiratory burst activity and neutrophil mediated endothelial cell damage (chapter 5.3). A clearly defined and generally accepted function of E-selectin as a mediator of neutrophil rolling behavior, being the first phase of neutrophil endothelial cell interaction during inflammation, has been worked out during the last years (18-20), and is described in chapter 1.
Specification of the role of TNF in neutrophil endothelial cell interaction, in relation to other inflammatory mediators, appeared to be more complex. TNF was more powerful in inducing

neutrophil respiratory burst activity and neutrophil mediated endothelial cell injury than other physiological neutrophil agonists such as IL-1, IL-8, and endotoxin (chapter 5.1 and 5.3). TNF-induced neutrophil activation depended on a direct effect of TNF on surface contacting neutrophils (chapter 5.1), without involvement of endothelial cell responses to TNF, such as endothelial IL-8 release (chapter 5.1, 5.3 and 5.4), but with involvement of endothelial cell PAF-expression in response to products of TNF-activated neutrophils (chapter 5.4). Based on these and other studies, such as a number of recent publications of Nathan et al. on the mechanism of prolonged neutrophil respiratory burst activity (21-24), and attempt can be made to specify the role of TNF in regulating neutrophil behavior during inflammation.

A characterization of the different phases in neutrophil behavior during inflammation, and arguments for a specific function for TNF as a 'secretagogue', a neutrophil agonist which triggers a highly adherent status and induces degranulation and strong respiratory burst activity, are depicted in chapter 1. Many details of this model still need to be clarified. The response of neutrophils to TNF-activation is regulated by the adhesive interaction with the environmentof the neutrophil. How actions of cytokines are integrated with information from adhesion-receptors remains a major question. Although the action of TNF on neutrophils offers one of clearest examples of the significance of the influence of matrix-proteins and adhesion-molecules in the response to cytokines, this influence is certainly not limited to neutrophils (reviewed in 25).

Intercellular communication seems to rely more principally on two arms: soluble, cytokine mediated, signals and the fibrous skeleton, composed of intracellular actin filaments, which are, via adhesion molecules, connected to filaments in neighbor-cells or to the extracellular connective tissue in which most cells are normally embedded. By this fibrous 'backbone' structure, cells are part of a larger organization. The composition and tightness of this structure might be a way by which responses of individual cells are coordinated.

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Chapter 3.1

TUMOR NECROSIS FACTOR-α (TNF) AND INTERLEUKIN 6 IN A ZYMOSAN INDUCED SHOCK MODEL

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Summary

TNF plays a central role in endotoxin or gram negative bacteria induced septic shock. Zymosan can elicit a 'septic shock' syndrome in rodents, in absence of endotoxin. TNF and IL-6 release in mice treated with zymosan was investigated. One hour after intra-peritoneal zymosan injection, maximal TNF-levels were measured in serum, followed by IL-6 peak-levels one hour later. Treatment with a monoclonal antibody against TNF lowered zymosan-induced mortality from 63% to 11.6%, while maximal IL-6 levels were lowered by 41%.

Mechanisms triggering zymosan induced cytokine-release in murine macrophages were analyzed in vitro. Cytokine-release was only slightly triggered by uncoated zymosan particles. 39% of TNF-release by macrophages appeared to be triggered by zymosan-bound activated complement. Maximal TNF release also required presence of natural antibodies against zymosan and zymosan activated serum. In contrast, maximal IL-6 release was reached upon stimulation with zymosan activated serum only, whilst presence of zymosan particles lowered this response.

We conclude that TNF is a crucial mediator in zymosan provoked 'septic shock'. TNF release can be incited by different immunological pathways, without a need for direct presence of endotoxins. Although IL-6 release during septic shock is partly dependent of TNF, in vitro trigger mechanisms for IL-6 and TNF differ remarkably.

Introduction

Zymosan, a product consisting of purified bakers yeast ghost-cells, can elicit a 'septic shock' syndrome in germ-free rats(15). This finding gave a new impulse to the discussion whether septic shock and multiple organ failure are linked obligatory to bacterial endotoxin, or represent a general overshoot in the activation cascade of the immune-system.

Recent investigations demonstrated a pivotal role for the monokine Tumor Necrosis Factor (TNF) or Cachectin in mediating these mechanisms. A single dose of recombinant TNF given to rats provokes hypothermia, hypotension, metabolic acidosis and lethargy, finally followed by death(33). Histological changes such as hemorrhagic lungs, interstitial edema, margination of neutrophils in several organs, intestinal necrosis and necrosis of renal tubuli are found in this model(20). Comparable results have been achieved in dogs and mice(4,35,24,21). These symptoms show great overlap with the phenomena seen after administration of bacterial endotoxin. Symptoms of endotoxin provoked septic shock could in turn be blocked effectively with antibodies directed against TNF(5,34).
TNF affects almost all cell types, inducing inflammatory as well as non-inflammatory responses. The pathways by which these responses are elicited are partly indirect, by provoking a release of other cytokines including IL-1, IL-6 and factors such as Platelet Activating Factor and prostaglandin E2 (7,39,9,12). Both IL-1 and TNF have been shown to stimulate neutrophil adhesion to endothelium(26), the first step in the process of tissue infiltration by neutrophils. The importance of this facet was demonstrated by preventing MOF and mortality in a rabbit hemorrhagic shock model with adhesion blocking antibodies(36).

Recent investigations have shown that part of the effects for which IL-1 was held responsible, were in fact provoked by IL-6(18). The exact effects of IL-6 in vivo are still largely unresolved. IL-6, also called hybridoma growth factor, B2 interferon or B-cell stimulatory factor 2, is a 26kD protein that has been shown to be related to acute inflammatory processes(19,1). IL-6 production is demonstrated for macrophages , fibroblasts, and a variety of other cell types(1,17, 31).
IL-6 has shown to have hepatocyte stimulating activity, thereby regulating the major acute phase protein response(13). Furthermore it acts as an activator of T-cell proliferation and is considered to induce synthesis of immunoglobulins by B-cells(19).

The putative involvement of TNF and IL-6 in zymosan provoked "septic shock" in mice was investigated. Serum levels of both cytokines were measured after a single intraperitoneal dose of zymosan. The effect of administration of an antibody directed against TNF, on survival and cytokine levels in mice treated with zymosan, was studied. Using an in vitro model, an attempt was made to reveal the pathways that contribute to the zymosan-elicited TNF and IL-6 production by murine macrophages.

Materials and Methods

Animals

Female Swiss-mice of an age of 10 weeks, weighing 24-29 grams, were obtained from Charles River Breeding laboratories (Heidelberg, FRG). They were maintained on standard laboratory chow and were allowed free access to food and water.

Reagents

Zymosan A (Sigma, St Louis, MO) for in vivo use, was suspended in highly liquid paraffine (Merck, Darmstad, FRG) 25g in 100ml. To obtain a homogeneous suspension, zymosan was thoroughly mixed with a small amount of paraffine in a mortar. Paraffine was used rather than saline to permit slower contact with the zymosan(15). For the in vitro use, zymosan was suspended in distilled water, heated at 100°C for 60 minutes and washed twice in saline. A stock solution of 500mg in 10ml of phosphate buffered saline (PBS) was stored at 4°C. TN3, a hamster monoclonal antibody specific for murine TNF and lymphotoxin, which inhibits the biological activity of these agents(32), was kindly provided by Drs Sheehan and Schreiber via Celltech, Slough, UK.

In vivo study

Mice were randomized in three groups on the day of the experiment. Group I (n=46) was given zymosan, 25mg in 1.0ml of paraffine. Group II(n=28) was given TN3, 500µg in 500µl PBS, four hours prior to the administration of zymosan. A third group of 10 mice served as a control group and was given only 1.0ml paraffine. All substances were administered by intra-peritoneal injection. Visual signs of illness, body-weight and mortality were registered twice daily.

In vitro study

Macrophages were obtained by peritoneal rinsing of ether anaesthetized mice with 10 ml RPMI 1640. The peritoneal macrophages were counted and concentrated to $5x10^5$ cells/ml in RPMI. Experiments were performed in quadruplicate, using 96 well cluster plates (Costar, Cambridge, MA). Each well received 100µl macrophage-suspension, 50µl zymosan-suspension (10mg/ml in RPMI) or RPMI, 50µl 50% murine serum in RPMI or RPMI. Serum was obtained freshly by cardiac puncture of mice anaesthetized with ether, and kept at 4°C. Zymosan-antibody free serum was made by adding an equal part zymosan suspension (20mg/ml in RPMI 1640) to the serum, followed by 30 minutes incubation on melting ice. Subsequently the serum was spun three times, in order to remove zymosan particles. Complement inactivation was performed by heating serum at 56°C for 30 minutes. Opsonized zymosan was prepared freshly by incubating zymosan with a 50% serum, 50% RPMI 1640 mixture (5mg in 1 ml) for 30 minutes on melting ice, followed by three wash-steps to remove serum. Zymosan activated serum was prepared by performing the same procedure at 37°C.

TNF elisa

96-Well immuno assay plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with TN3 (5µg/ml). Plates were blocked with 1% (wt/vol) bovine serum albumin (BSA)(Sigma, St Louis, MO) in PBS for one hour at room temperature.

After four washings with wash-buffer (consisting of PBS, 0.1% BSA, 0.1% Tween 20) test samples were added to the plate for one hour at room temperature. A standard titra-

tion curve was obtained by making serial dilutions of a known sample of recombinant murine TNF (Genzyme, Cambridge, MA) in medium identical to the test sample. Next, plates were washed four times with wash-buffer and incubated with rabbit anti-mouse TNF immune serum (Genzyme) and peroxidase conjugated goat anti-rabbit IgG (Jackson, WestGrove, PA).

After adding the substrate (o-Phenylene-diamine, 0.43 mg/ml, Sigma) to the plates for 10 minutes, the color-reaction was stopped with 1.0 M H_2SO_4 and photospectrometry (492 nm) was performed using a micro ELISA autoreader (Flow, Irvine, UK).

The ELISA has a lower detection limit of 150 pg/ml.

IL-6 bioassay

The IL-6 dependent B9 cell-line(1), kindly provided by Dr L. van Aarden (CLB, Amsterdam, The Netherlands) was used to determine IL-6 levels. Calculations were performed as described by S. Gillis et al(14).

In short: cells were cultured in 10% bovine calf serum (BCS) (Hyclone, Logan, Utah), 100 units/ml Penicillin G and 100 µg/ml streptomycin in RPMI 1640 medium, containing 5 units IL-6/ml and grown in 96-well serocluster plates (Costar).

Cells were washed twice to remove IL-6 at the beginning of the assay, then brought to $5x10^4$ cells/ml and transferred to RPMI 1640 containing 10% BCS and gentamicin.

Serial dilutions of the test samples were added to the wells. A standard titration curve was made with recombinant human IL-6 sample kindly provided by Dr L. van Aarden. After 68 hours of culture at 37 °C, cells were incubated for four hours with 0.25 µCi ^{3}HTdR (specific activity 5.0 Ci/mM).

Cell-proliferation was defined by measuring ^{3}HTdR uptake in a liquid scintillation counter.

Statistics

Statistical significance was determined using the Students *t* test and the chi square test. A p-value less than 0.05 was considered to be significant.

Results

In vivo study

Shortly after intra-peritoneal injection of 25 mg zymosan, mice became lethargic and stopped eating. Within hours they developed symptoms like rugged fleece, tachypnoea, swollen mucous membranes and diarrhoea.

In a group of 20 animals, IL-6 and TNF serum concentrations were measured *(Fig.1)*. Both cytokines appeared rapidly after zymosan injection in the serum. Peak levels were reached at one hour for TNF, at two hours for IL-6, followed by a steep decline.

Treatment with monoclonal antibodies against TNF, four hours before administration of zymosan, resulted in lowering the peak-level of IL-6 serum-concentration from 1160 units/ml to 690 units/ml, whilst 500 µg of TN3 showed adequate to prevent measurable TNF serum-concentrations. Serum samples of mice in the paraffine treated control group, showed elevation of neither IL-6 nor TNF level.

Investigations about the influence of monoclonal antibodies against TNF on zymosan-induced mortality were performed by comparing three groups of mice; group A (10 mice) who received 1.0 ml paraffine, group B (46 mice) who received 25 mg zymosan suspended in 1.0 ml paraffine, and group C

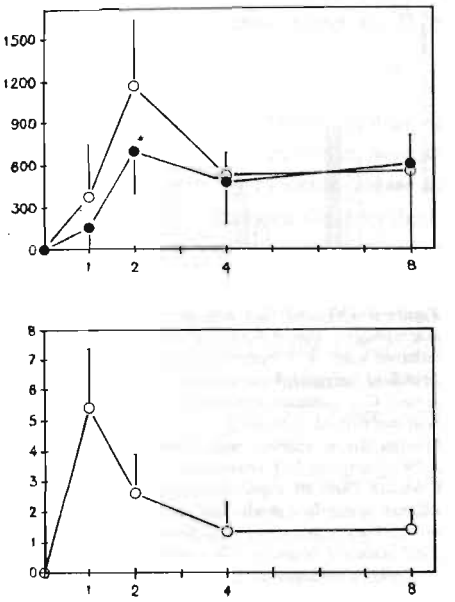

Figure 1. IL-6 (A) and TNF (B) serumconcentrations during first 8 hours after intra-peritoneal administration of 25 mg zymosan. Open circles represent cytokine-levels in zymosan-treated mice, filled circles represent cytokine-levels in zymosan plus TN3 treated mice. During the first eight hours TNF serumlevels in TN3 treated mice were below detection limit. Data are expressed as means (n=4) ± SD.
** P=0.137 compared with zymosan only T=2h.*

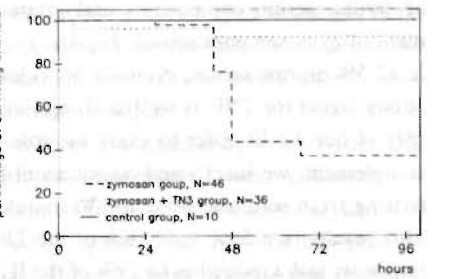

Figure 2 Influence of administration of antibodies directed against TNF on mortality in 25 mg zymosan treated mice. In the period between 4 days and the end of the observation period at 13 days after zymosan administration, no mortality was recorded.
** Difference in mortality between zymosan treated mice with and without TN3 pretreatment is highly significant, ($p<0.001$).*

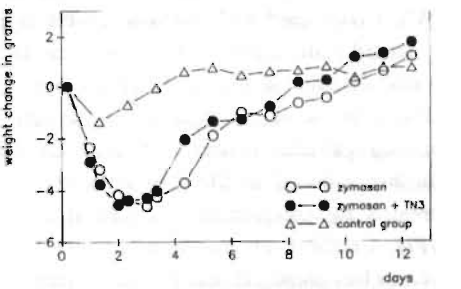

Figure 3 Change body-weight during first 12 days after treatment with zymosan, intervention with TN3, or paraffine only (control group). Data are expressed as means of the mice shown in Figure 2. The zymosan/paraffine administration directly before the first measurement resulted in a rise in body-weight of 0.9 grams.

(26 mice) who received 500 µg antiserum against TNF, four hours before zymosan-administration.

After two days of severe illness in group B and C (in both groups a weight-loss of about 4.5 grams was recorded), 63.0% of the mice in group B died. Mortality in group C was reduced to 11.6%, which implicates that treatment with TN3 prevents more than 80% of zymosan induced mortality in this situation *(Fig. 2)*. Weight recovery began three days after zymosan-injection, appearing slightly faster in group C as in the survivors of group B, and was complete after 11 days *(Fig. 3)*.

In vitro study

Production of both cytokines by freshly isolated peritoneal macrophages was measured after 6 hours of exposure to zymosan at

37°C. In this model we studied the influence of serum, serum components and pretreatment of zymosan with serum *(Fig.4)*.
In 12.5% murine serum, zymosan provides a strong signal for TNF as well as IL-6 release *(Fig. 4 bar A)*. In order to study the role of complement, we inactivated complement by heating fresh serum to 56°C for 30 minutes. This results in a 56% reduction of the TNF response, and a reduction to 19% of the IL-6 response *(Fig. 4 bar B)* on zymosan stimulation in presence of normal serum (standard stimulation).

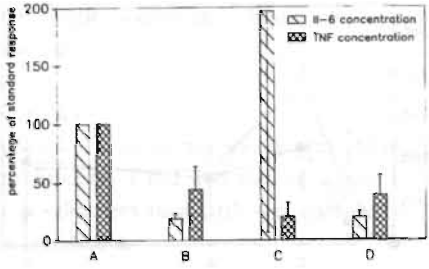

***Figure 4** TNF and IL-6 release by murine peritoneal macrophages, after 6 hours culture. Macrophages were cultured with: A = zymosan in untreated fresh serum (standard release);B = zymosan in heat inactivated serum; C = zymosan activated serum; D = zymosan in heat inactivated, antibody free serum. Spontaneous cytokine release in cultures with untreated serum reached 3.7% of standard TNF release and 19.4% of standard IL-6 release. Data are expressed as means (n=4) ± S.D. in relation to cytokine production in cultures with zymosan in 12.5% untreated serum (standard response). This standard response (expressed as 100%) reaches 18 ng/ml TNF and 9,600 units/ml IL-6.*

The role of zymosan activated serum and serum opsonized zymosan particles was tested by separating serum and zymosan particles after an incubation period of 30 minutes. When compared with cytokine levels after standard stimulation, TNF response on activated serum was only 20% *(Fig.4 bar C)*. The response of macrophages to the serum treated particles reached 70%, which was further reduced to 20% if particles were treated by complement inactivated serum *(Fig. 5 respectively bar A and bar B)*. Thus, it can be concluded that the major stimulus for TNF-production is formed by zymosan particles opsonized by complement.

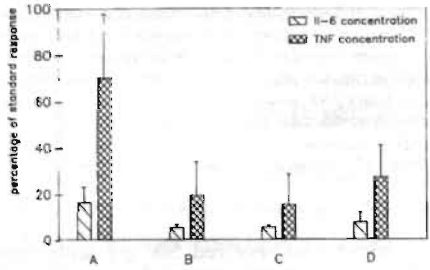

***Figure 5** TNF and IL-6 release by macrophages after exposure to zymosan, in serumfree medium. Macrophages were cultured 6 hours at 37 °C with: A = zymosan opsonized with normal serum; B = zymosan opsonized with heat inactivated serum; C = unopsonized zymosan; D = medium without zymosan. Data are expressed as means (n=4) ± S.D. in relation to the standard response (see Fig. 4).*

The IL-6 response on complement opsonized zymosan particles does hardly exceed spontaneous release *(Fig. 5 respectively bar A and bar D)*. Activated serum without zymosan particles however appeared to trigger IL-6 levels nearly twice as high (196%) as in the same medium with zymosan particles still present *(Fig. 4 respectively bar C and bar A)*. This value showed the highest variation in a series of experiments, which appeared to be dependent on low contamination with zymosan particles caused by insufficient spinning-out of these particles. Therefore we conclude that in this model IL-6 release is mainly induced by zymosan activated serum.

Even small amounts of zymosan particles appear to have a negative effect on IL-6 release.

To investigate whether natural occurring antibodies directed against zymosan, have an important function in the cytokine release as measured above, we removed these antibodies from heat inactivated serum (removing antibodies from normal serum would also diminish complement activity) as described. Zymosan particles in such antibody depleted and heat inactivated serum induced a TNF-response which was only 5% lower compared to TNF-responses upon zymosan in heat inactivated serum with antibodies still present *(Fig. 4 respectively bar D and bar B)*. IL-6 release upon zymosan in heat inactivated serum was not influenced by antibody-removal.

Discussion

In this study, we show that one hour after intra peritoneal zymosan administration in mice, a peak-concentration of TNF can be measured in serum. Furthermore, circulating TNF can be reduced for almost 100% by injecting a monoclonal antibody directed against TNF.

This inhibition of biologically active TNF is shown to be associated with a reduced severeness of the zymosan provoked illness as reflected by significantly lower mortality rates. Therefore we conclude that TNF can be considered to be a pivotal mediator in zymosan provoked shock, a role which TNF also is thought to play in sepsis(5,34,10,3).

IL-6 has been measured in serum from patients with meningococcal septic shock (37), in serum of renal transplant recipients(23) and in synovial fluids of patients with rheumatoid arthritis(16). IL-6 serum levels might be a good grade-indicator of the shock-syndrome provoked by zymosan. We measured IL-6 serum levels shortly after zymosan injection.

Peak levels were reached one hour after TNF had its maximal serum concentration. Administration of a monoclonal antibody directed against TNF reduced this peak by 41%. These findings indicate that IL-6 may in part be provoked by TNF or by TNF related events.

Similar data were obtained after administration of bacterial endotoxin in mice (manuscript submitted for publication).

However, the data also indicate that other important IL-6 triggering mechanisms exist. Furthermore, it appeared that animals receiving TNF-neutralizing antibodies developed severe symptoms of illness, indicating that besides TNF other factors are involved in the pathogenesis of zymosan induced shock.

The mechanisms which mediate the release of TNF and IL-6 after exposure to zymosan were investigated in vitro experiments.

Zymosan consists of ghost cells derived from bakers yeast and contains primarily polysaccharides without detectable protein(25). So far three mechanisms are known, by which zymosan can trigger an immune response. Firstly, normal serum contains natural IgG immunoglobulins which bind to zymosan(30). Complexes formed this way activate the classical pathway of the complement system, and may elicit TNF-release by cross-linking macrophage Fc-receptors(11).

Secondly, receptor mediated binding and ingestion of the zymosan particle can occur. Complement receptor 3, which is present on macrophages as well as granulocytes, has

two binding-sites: a lectin-like region, with direct binding-potential to zymosan, bacterial endotoxin etc., and an iC3b specific binding site(38). The latter function results, in synergistic cooperation with CR1, in increased binding and ingestion of complement-opsonized particles(28). Thirdly, the binding of C3b to the zymosan-surface results in increased conversion of C5 to C5a(29), which has been shown to trigger monocyte TNF-release(22).

Figure 6 summarizes the different pathways by which zymosan triggers macrophage TNF and IL-6 release in medium containing 12.5% serum, as they can be deduced from our in vitro experiments.

TNF release appeared to be dependent on all three groups of mechanisms cited above, although the effects that can be contributed to macrophage Fc-receptor crosslinking seems to be small and is therefore not significant in this series of experiments. Surprisingly, serumfree culture of macrophages in presence of bare zymosan particles does not trigger more TNF release than control cultures do.

An explanation for this finding might be that the very same mechanism that underlies TNF release induced by bare zymosan particles, is also triggered by plastic surfaces not coated with serum proteins.

The relevance of this specification of trigger mechanisms for macrophage TNF release might be, that the connection between bacterial endotoxin and TNF release is not as direct and unique as is often thought.

Parallel to this, also the septic shock syndrome might, for as far as it is related more to systemic TNF levels than systemic endotoxin challenge, be due to a general activation of immunological systems, more than to bacterial infections directly.

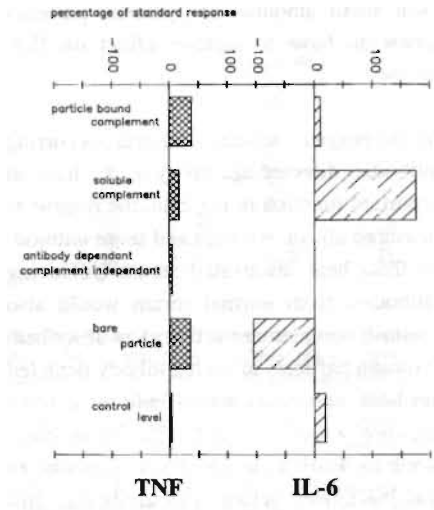

Figure 6 *Contributions of particle bound complement, soluble complement, antibody dependent and complement independent activation, bare zymosan particles and spontaneous release in the standard macrophage IL-6 and TNF response (see Fig. 4). Data shown here are interpretations which can be deduced directly from the data as shown in Fig.4*

The IL-6 triggering capacity of the different mechanisms investigated here, showed a completely different pattern *(see Fig. 6 for summarized data)*. Whereas several pathways add synergistically to incite macrophage TNF release, highest IL-6 levels are reached upon culturing macrophages in zymosan activated serum.

The induction of IL-6 release by particle bound complement is very low compared to serum containing activated complement.

Fc-receptor mediated triggering of macrophages fails to induce extra IL-6 release in this model. The linkage between IL-6 release and soluble activated complement is stressed

by the finding that presence of zymosan particles even lowers IL-6 release. Moreover, serumfree culture of macrophages with zymosan particles opsonized with unheated serum results in a lower IL-6 release than the spontaneous release measured in 12.5% serum. TNF levels, in contrast, reached up to 51 ng/ml in the same cultures (unheated serum opsonized zymosan particles in serumfree medium), whilst spontaneous release in serum was 0.9 ng/ml.

Thus, although there are several reports of IL-6 release induced by TNF exposure in vivo and in vitro (39,8,31,6), the relationship between these two cytokines might be much more complex than a simple causal system. TNF and IL-6 but also TNF and IL-1(27) can be induced separately.

It appears that these cytokines, which have closely linked biological effects, each have their own pattern of triggers or conditions which regulate their release. Compared to other mainly quantitative humoral systems, like the clotting cascade or the complement system, relations in the network of cytokines might have much more distinguished qualitative aspects.

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Chapter 3.2

IL-6, IL-8 AND TNF PRODUCTION BY CYTOKINE AND LIPOPOLYSACCHARIDE-STIMULATED HUMAN RENAL CORTICAL EPITHELIAL CELLS *IN VITRO*

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Abstract

The capacity of renal epithelial cells to produce IL-6, IL-8 and TNF was investigated. Cultures of explanted human renal cortical epithelial cells (RCEC) were established, and cytokine-release and mRNA expression by these cells were measured. IL-6, IL-8 and TNF release were measured after stimulation with IL-1β, TNF-α, LPS and the phorbol esther PMA. All these agents were found to induce increased release of all three cytokines. Whilst no spontaneous TNF-release occurred, IL-6 and IL-8 were continuously released by non-stimulated RCEC cultures. IL-1β was the most potent trigger enhancing both RCEC cytokine release and expression of IL-6, IL-8 and TNF mRNA. Indomethacin, budesonide, cyclosporin and FK 506 were tested for their influence on RCEC cytokine release. Only the steroid budesonide appeared to reduce both spontaneous and IL-1β induced cytokine release.

Our data demonstrate stimulus specific release of IL-6, IL-8 and TNF by RCEC, and suggest that cytokine cell-to-cell communication may be important in regulating inflammatory processes in the kidney.

Introduction

Renal allograft rejection, and a number of auto-immune syndromes can cause life-threatening renal injury. Cytokines are generally considered to be mediators responsible for immune-injury leading to progressive cell-death and organ disfunction. Several lines of evidence support such a role for TNF in renal injury.

In vitro studies demonstrated that TNF triggers toxicity of neutrophils (1, 2) and eosinophils (3), functions as a co-stimulatory factor for T-cells (4, 5), but can also induce pro-inflammatory changes in endothelial cells (6, 7), renal mesangial cells (8, 9) and other non hematopoietic cells.

In vivo, infusion of TNF was shown to induce glomerular damage in rabbits (10), to potentiate renal damage caused by ischemia in rats (11), to accelerate renal disease and mortality in a murine model for lupus nephritis (12) and to cause exacerbation of antibody-mediated glomerular injury in rats (13). Furthermore, a positive correlation of renal expression of TNF mRNA with disease activity in mice with lupus nephritis (12) and rats with immune-complex glomerulonephritis has been described (14).

In a rat model for nephrotoxic serum nephritis, inhibition of renal injury by administration of anti-TNF antibodies was demonstrated (15). In humans, presence of TNF and IL-6 in serum (16-20) and increased renal TNF and IL-6 mRNA expression (21, 22) has been demonstrated during renal allograft-rejection. Besides suggesting involvement of TNF in inflammatory renal injury, the presence of mRNA also raised the possibility that renal cells would produce and secrete TNF and IL-6. TNF-production, both *in vitro* by monocytes or *in vivo*, is accompanied by production of IL-6 and IL-8.

Although IL-6 and IL-8 have not been described to be directly responsible for tissue-injury, these cytokines act in concert with TNF to modulate inflammatory processes. IL-6 and IL-8 are released by hemopoietic as well as non-hemopoietic cells such as endothelial cells and fibroblasts in response to bacterial LPS, cytokines and other stimuli (7, 23-26).

IL-6 is a multi-potential inflammatory cytokine which induces acute phase protein synthesis by the liver (27), maturation of B-cells (28) and activation of T-cells (29). IL-6 has, however, also an anti-inflammatory role by down-regulating TNF-release upon LPS-challenge (23). IL-8 mainly acts on neutrophils; IL-8 regulates transendothelial migration (26, 30), and induces neutrophil activation (31).

In this study, we investigated whether cultured human RCEC can be triggered to release IL-6, IL-8 and TNF. Furthermore, we studied the regulation of the release of these cytokines.

Material and methods

Reagents

rh TNF-α was kindly provided by BASF/Knoll AG. (Ludwigshafen, FRG). rhIL-1β was a kind gift of Dr. S. Gillis (Immunex, Seattle, WA). LPS (phenol extract of E. Coli 055:B5) and PMA were purchased from Sigma (St. Louis, MO).

RCEC culture

RCEC explant cultures were obtained using previously described techniques (32). Briefly, small biopsies from the human renal cor-

tex were obtained from kidneys not suitable for transplantation for technical reasons. The capsula was removed and the cortex-tissue was cut into small pieces, which were incubated in 0.25% trypsin for 25 minutes at 37°C. The mixture was then filtered through one layer of sterile gauze to remove glomeruli, the filtrate was centrifuged, and the pellet was resuspended in RPMI-1640 (Gibco Europe, Paisley, Scotland) supplemented with 10% heat-inactivated bovine calf serum (BCS) (Hyclone, Logan, UT) and antibiotics.

The mixture was placed in fibronectin coated (fibronectin kindly provided by Dr. J. van Mourik, CLB, Amsterdam, The Netherlands) tissue culture flasks (Costar, Cambridge, MA). When confluent, the monolayers were subcultured by harvesting the cells by addition of trypsin-EDTA (0.05%, 0.02% in Ca^{++} Mg^{++} free PBS), and redistributed to new flasks at a 1:3 subculture ratio. Cells were redistributed 3 times before use in the experiments.

RCEC characterization

The cultured renal cortical cells showed a pavement-like monolayer morphology which remained stable for 5 to 6 redistribution cycles. Functional characteristics of subcultures gained by above described procedures have been extensively studied, showing that these cells retain a number of functional characteristics typical of the proximal tubule (32). The cultured RCEC were characterized by immunofluorescence examination.

No cells showed positive staining after incubating TNF-activated monolayers with an anti E-selectin mAb, excluding endothelial cell contamination. Also, no cells showing positive staining after incubation with an anti-CD14 mAb, excluding contamination with mononuclear phagocytes. Bright fluorescence, consistent with intermediate filaments, was seen for all cells using an anti-cytokeratin monoclonal antibody.

This monoclonal antibody staining pattern is consistent with a homogenous primary culture of cells of epithelial origin.

Culture of human umbilical vein endothelial cells (HUVEC) and human dermal fibroblasts.

HUVEC were obtained by collagenase treatment of the human umbilical vein. The cells were cultured in fibronectin-coated tissue culture flasks in RPMI-1640, supplemented with 10% heat-inactivated human serum, 10% BCS, 50 µg/ml heparin (Sigma), 30 µg/ml endothelial growth supplement (Collaborative Research Incorporated, Bedford, MA) and antibiotics.

Endothelial cells were characterized by their pavement-like monolayer morphology and by positive staining with an anti E-selectin mAb after 4 h incubation with TNF. Cells of passage 3 to 4 were used for the experiments.

Human dermal fibroblasts (kindly provided by the department of Human Genetics, University of Limburg, The Netherlands) were propagated in RPMI-1640 supplemented with 10% BCS and antibiotics in fibronectin coated tissue culture flasks and characterized by their spindle-like morphology. Endothelial cell contamination was absent, as concluded from negative staining with an anti E-selectin mAb after 4 h incubation with TNF. Cells of passage 6 to 10 were used for the experiments.

Induction of cytokine release

HUVEC, RCEC, and human fibroblasts were seeded at 10^4 cells/well in fibronectin-coated 96-well flat-bottom tissue culture plates (Costar) in culture medium, one day prior to stimulation.

The cells were rinsed twice with RPMI-1640 and the medium was replaced by the appropriate agents in RPMI-1640 supplemented with 10% BCS and antibiotics. Media and preparations of TNF, IL-1β and PMA were tested for endotoxin contamination by the chromogenic limulus amoebocyte lysate assay (Coatest, Kabi Diagnostica, Nyköping, Sweden), and were found to contain less than 5 pg/ml LPS in final solutions. Supernatants were harvested and kept at -20°C until use in the IL-6, IL-8 and TNF ELISA.

IL-6, IL-8 and TNF ELISA

The culture supernatant IL-6, IL-8 and TNF concentrations were determined using previously described sandwich-ELISA for IL-6 (33), IL-8 (34) and TNF (35). In short, 96-well immuno maxisorp plates (Nunc, Roskilde, Denmark) were coated with cytokine-specific murine mAb; for IL-6 a newly developed IL-6 specific mAb 5E1 was used. rhIL-6, a generous gift from Prof. Dr. W. Sebald (Physiologisch-Chemisches Institut der Universität, Würzberg, FRG), rhIL-8 and rhTNF-α were used for standard titration curves. Test samples were added.

Next polyclonal rabbit anti-human IL-6 antiserum and polyclonal rabbit anti-human TNF-α were added, followed by peroxidase conjugated goat anti-rabbit IgG (Jackson, Westgrove, PA) for the IL-6 and TNF-ELISA respectively. Phosphatase conjugated polyclonal goat anti-human IL-8 was used for the IL-8 ELISA. O-Phenylene-diamine (Sigma) was added as a substrate for peroxidase and p-nitrophenyl phosphate (Sigma) as a substrate for phosphatase. Photospectometry was performed at 492 nm (IL-6 and TNF ELISA) and 405 nm (IL-8 ELISA). The ELISA lower detection limits were 10 pg/ml IL-6, 100 pg/ml IL-8 and 10 pg/ml TNF-α.

Preparation and analysis of RNA

RCEC were grown and stimulated as described in 150 cm^2 tissue culture flasks (Costar). Total cellular RNA was isolated as described (36). RNA samples were treated as recommended by Amersham and applied onto a N^+ Membrane (Amersham, Buckinghamshire, UK), using a Biodot apparatus (Biorad, Richmond, CA). cDNA probes were labeled with ^{32}P dCTP using the random primer labeling kit of Boehringer Mannheim (Boehringer Mannheim, Mannheim, FRG) and hybridized to the blot (10^6 cpm/ml) as described (37).

The IL-6 probe (1.3 kb HIII-EcoRI fragment) was kindly provided by Dr. Aarden (CLB, Amsterdam, Netherlands), the IL-8 probe (0.35 kb EcoR1 fragment) was kindly provided by Dr. I. Lindley (Sandoz, Wien, Austria) and the actin probe (1.3 Pst fragment) was kindly provided by Dr. Berkvens (University of Leiden, Leiden, The Netherlands). Labeled bands were visualized by autoradiography.

RESULTS

RCEC produce IL-6, IL-8 and TNF in response to cytokines, LPS and PMA

IL-6, IL-8 and TNF release by cultured explanted RCEC from four different donors were measured after 18 h of stimulation with different agents. In absence of stimulus, nanograms per milliliter of both IL-6 and IL-

Table 1: IL-6, IL-8 and TNF release by RCEC

	unstimulated			100 ng/ml TNF		1 µg/ml LPS			100 U/ml IL-1			100ng/ml PMA		
Donor:	IL-6	IL-8	TNF	IL-6	IL-8	IL-6	IL-8	TNF	IL-6	IL-8	TNF	IL-6	IL-8	TNF
1.	14±2	2.6±0.3	0	17±2	3.1±0.5	18±2	4.0±0.5	0.03±0.02	72±8	23±4.0	0.21±0.03	56±8	12±2.1	0.04±0.02
2.	10±1	2.1±0.4	0	18±1	3.8±0.7	11±2	5.7±0.2	0	66±7	17±0.9	0.01±0.01	43±6	16±1.7	0
3.	15±2	3.3±0.3	0	20±1	4.4±0.4	18±1	7.3±0.5	0.01±0.01	84±5	46±0.8	0.26±0.03	59±3	40±2.6	0.03±0.01
4.	17±1	3.6±1.0	0	22±1	5.2±1.0	19±1	9.6±2.0	0	90±4	37±3.2	0.02±0.01	57±5	31±2.8	0.01±0.01

RCEC from four different donors were cultured to a density of 2×10^4 cells/well, ans stimulated with the indicated agents in one experiment. Supernatant-samples were collected after 18 hours of culture, and IL-6, IL-8 and TNF were measured in identical samples. Data are expressed in ng/ml as mean ± SD from four measurements.

8 were present in the supernatant medium, implicating a release of more than 1 ng of IL-6 and IL-8 per 10^6 cells per hour *(Table 1)*. Ongoing spontaneous IL-6 and IL-8 release was found in the primary *in vitro* culture, and remained stable up to six redistribution cycles. Presence of IL-1β, and to a lesser extend presence of TNF and PMA during the 18 h incubation period induced clear increases in IL-6 and IL-8 levels *(Table 1)*.

In contrast to IL-6 and IL-8, no spontaneous TNF release was measured. In part of the cultures, stimulation with IL-1β and PMA induced TNF release. TNF release was, in comparison to IL-6 and IL-8 release, lower and showed strong variation between cells from different donors *(Table 1)*.

IL-6, IL-8 and TNF supernatant concentrations showed a near-linear increase during the first 24 h of stimulation, with a lag-peri-

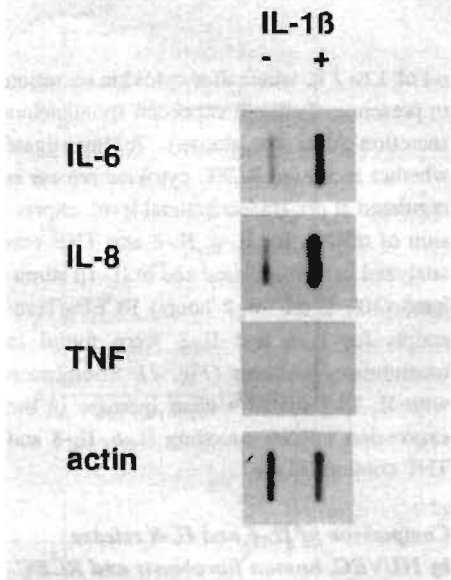


Figure 1: IL-1β increases IL-6, IL8 and TNF mRNA expression in RCEC cultures.
mRNA was isolated from untreated RCEC (left panel) and RCEC stimulated for 3 hours with 100 U/ml IL-β. Parallel blots were run and hybridized with actin probe to ascertain that total RNA amounts of different samples were identical.

Table 2: The influence of immuno-suppressive agents on IL-1 induced cytokine release.

	HUVEC		Fibroblasts		RCEC		
	IL-6	IL-8	IL-6	IL-8	IL-6	IL-8	TNF
No agent	5.4 ± 0.9	15.0 ± 1.1	25.0 ± 3.7	20.2 ± 2.0	25.5± 4.7	11.0 ± 1.3	0.27 ± 0.04
Indomethacin 10^{-6}M	5.4 ± 0.6	14.4 ± 1.5	17.9[1]± 1.5	21.0 ± 5.9	26.0± 1.0	10.6 ± 1.6	0.32 ± 0.06
Budesonide 10^{-7}M	4.5 ± 0.7	12.8[1]± 1.6	5.6[2]± 0.4	11.9[1]± 2.1	19.3[1]± 3.6	10.3 ± 1.8	0.13[1]± 0.03
Cyclosporin 10^{-6}M	4.3[1]± 0.1	14.2 ± 2.1	21.8 ± 0.9	21.0 ± 4.4	22.7± 4.2	10.4 ± 1.8	0.24 ± 0.06
FK 506 10^{-6}M	5.0 ± 0.5	14.5 ± 2.3	24.0 ± 0.5	20.5 ± 5.2	24.2± 2.7	10.4 ± 2.7	0.27 ± 0.03

Cells were stimulated with 100U/ml IL-β for 18 hours. Immuno-suppressive agents were added together with IL-1. IL-6, IL-8 and TNF release were measured in identical samples. Data are given in ng/ml as mean and SD of quadruplicates.
[1] $p<0.05$ and [2] $p<0.01$, as compared to cytokine release under identical circumstances but in absence of the immuno-suppressive agent (determined by the unpaired, two-tailed Student's t-test).

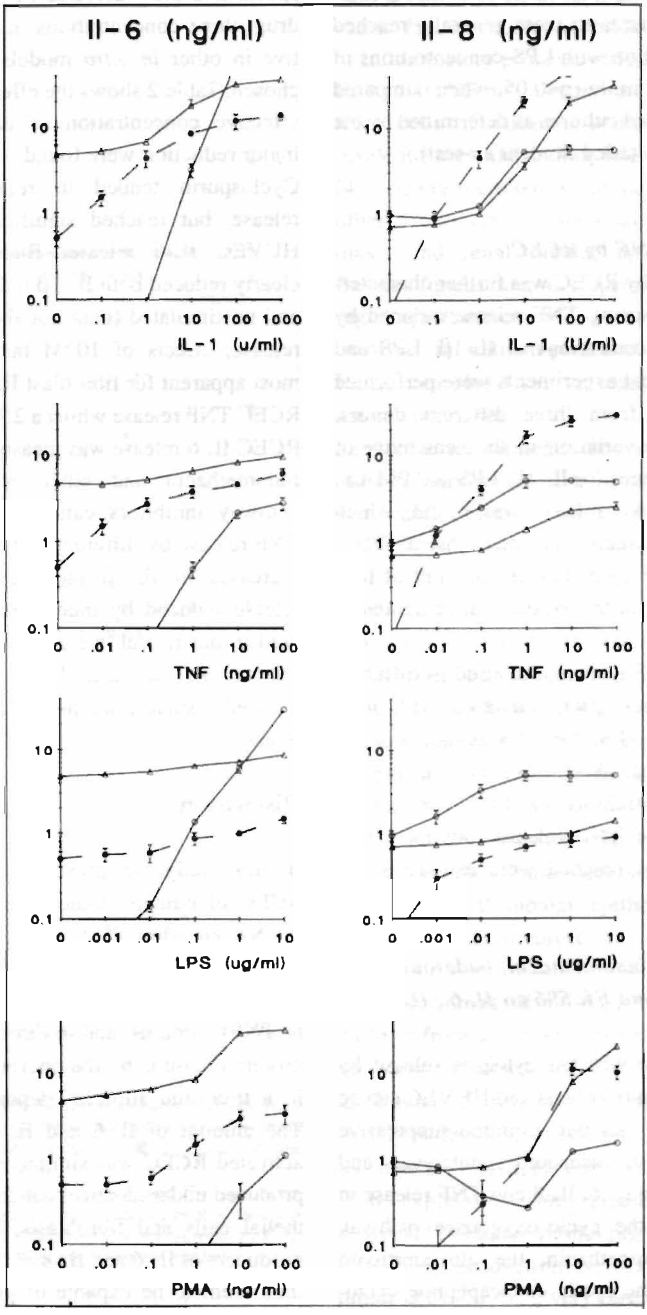
od of 1 to 2 h, whereafter cytokine secretion in presence of stimuli exceeded spontaneous secretion (data not shown). To investigate whether increased RCEC cytokine release is regulated at pre-transcriptional level, expression of mRNA for IL-6, IL-8 and TNF was analyzed in unstimulated and in IL-1β stimulated (100 U/ml for 2 hours) RCEC. Transcripts for IL-6 and IL-8 were found in unstimulated cultures *(Fig. 1)*. Stimulation with IL-1β induced a clear increase in the expression mRNA encoding IL-6, IL-8 and TNF content *(Fig. 1)*.

Comparison of IL-6 and IL-8 release by HUVEC, human fibroblasts and RCEC

Since HUVEC and fibroblast are well known producers of both IL-6 and IL-8, we compared the induction profile of IL-6 and IL-8 release by these cells and by RCEC. The influence of 18 h stimulation with different concentrations of TNF, LPS, IL-1β and PMA on IL-6 and IL-8 release was measured *(Fig. 2)*. Without stimulation, RCEC secreted both IL-6 and IL-8, HUVEC secreted IL-8 only, whereas fibroblast secreted majorly IL-6.

The three cell-types showed an increased IL-6 and IL-8 release in response to all 4 stimuli *(Fig. 2)*. The highest amounts of IL-6 and IL-8 release were seen after stimulation with 100 to 1000 U/ml IL-1β. RCEC and fibroblasts responded, in contrast to HUVEC, very poorly to stimulation with LPS. A small increase in both RCEC IL-6 and IL-8 release was, however, found throughout all experi-

Figure 2: IL-6 and IL-8 release by HUVEC (O), fibroblasts (●) and RCEC (▲) after stimulation with the indicated agents.
Cells were stimulated for 18 hours and IL-6 and IL-8 concentrations were determined in supernatant-samples. Data are expressed as mean ± SD from four measurements. If SD bars are not shown, they fall within the symbol.


Il-6 (ng/ml)
Il-8 (ng/ml)
IL-1 (u/ml)
IL-1 (U/ml)
TNF (ng/ml)
TNF (ng/ml)
LPS (ug/ml)
LPS (ug/ml)
PMA (ng/ml)
PMA (ng/ml)

ments in which LPS was used in this study. Significant increases were generally reached after stimulation with LPS-concentrations of 100 ng/ml or more ($p<0.05$, when compared to unstimulated cultures as determined by the unpaired two-tailed Student's *t*-test).

Release of TNF by RCEC

TNF release by RCEC was further characterized by measuring TNF release induced by different concentrations of IL-1β, LPS and PMA. Identical experiments were performed with RCEC from three different donors. Considerable variation in the sensitivity of different cultures for IL-1β, LPS and PMA in respect to TNF release was found, which probably explains the near-absent TNF-release found by cells from two out of four donors tested in the experiment presented in table 1.

Maximal TNF release measured in different RCEC cultures after activation with high amounts of IL-1 and PMA was more consistent and reached about 1 to 1.5 ng/ml *(Fig. 3)*. High concentrations of LPS consistently induced some TNF release, but the TNF-concentrations reached were usually below 100 pg/ml.

Influence of indomethacin, budesonide, cyclosporin and FK 506 on IL-6 , IL-8 and TNF release

To investigate whether cytokine release by RCEC, human fibroblast and HUVEC can be regulated by known immuno-suppressive compounds, we measured spontaneous and IL-1β induced IL-6, IL-8 and TNF release in presence of the cyclo-oxygenase pathway inhibitor indomethacin, the glucocorticoid budesonide, the cyclic endecapeptide cyclosporin and the macrolide FK 506. For each drug, three concentrations in a range effective in other *in vitro* models (38-41) were chosen. Table 2 shows the effects of the most effective concentration of each drug. No major reduction were found.

Cyclosporin tended to reduce cytokine-release, but reached significance only for HUVEC IL-6 release. Budesonide more clearly reduced both IL-1β induced *(Table 2)* and unstimulated (data not shown) cytokine release; effects of 10^{-7}M budesonide were most apparent for fibroblast IL-6 release and RCEC TNF release whilst a 25% reduction in RCEC IL-6 release was measured *(Table 2)*.

Indomethacin and other cyclo-oxygenase pathway inhibitors can increase IL-1 and TNF release by different cell-types (40, 42). Increases in IL-1β induced RCEC TNF-release induced by indomethacin, as measured in our model, were to small to be conclusive. In contrast, 10^{-6}M indomethacin induced a small reduction in fibroblast IL-6 release.

Discussion

In this study we presented evidence that RCEC of human tubule origin can express mRNA encoding IL-6, IL-8 and TNF, and secrete these proteins. RCEC were able to respond to primary (LPS), secondary (IL-1β or TNF) stimulus, and to direct activation of protein kinase C by the phorbol esther PMA in a time and stimulus dependent fashion. The amount of IL-6 and IL-8 released by activated RCEC was similar to the amounts produced under identical conditions by endothelial cells and fibroblasts, known strong producers of IL-6 and IL-8 (7, 24-26). RCEC thus seem to be capable of producing rele-

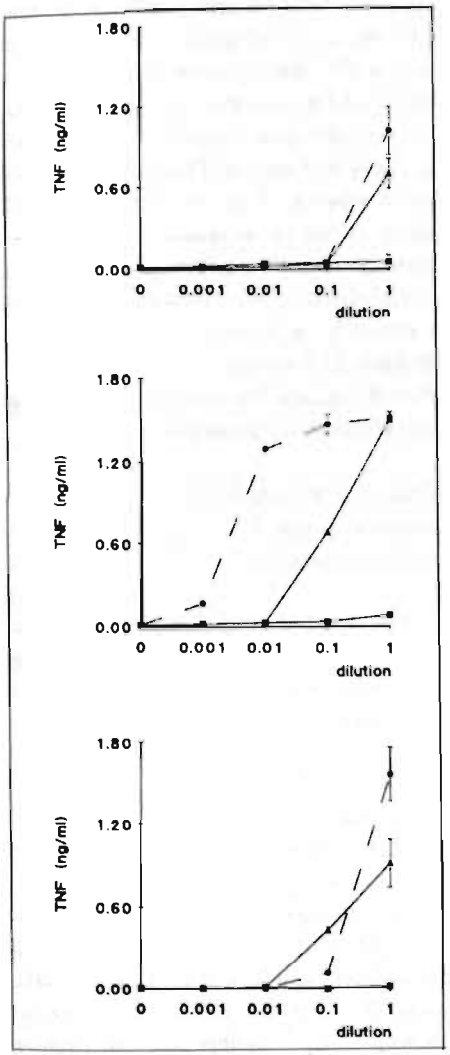


Figure 3: TNF release by three cultures of RCEC from different donors after stimulation with serial dilutions of IL-1β (●, 1 = 1000 U/ml), LPS (■, 1 = 1 μg/ml) and PMA (▲, 1 = 100 ng/ml).
Cells were stimulated for 18 hours and TNF concentrations were determined in supernatant-samples. Data are expressed as mean ± SD from four measurements. If SD bars are not shown, they fall within the symbol.

vant amounts of IL-6, IL-8 and TNF, implicating that, in the human kidney, the 'tuning' of inflammatory processes triggered by IL-6, IL-8 and TNF may depend on contributions from cells of different sources, including resident interstitial mononuclear phagocytes (43), newly recruited mononuclear cells and other leukocytes, endothelial cells, fibroblasts and renal tubular epithelial cells. Besides functioning as a specific target during allograft rejection (44, 45), which can be involved in antigen-presentation (46, 47), RCEC can play a role in regulating inflammatory processes.

Leukocyte-recruitment, mediated by TNF-induced expression of adhesion molecules, neutrophil migration, mediated by an IL-8 gradient, and activation of lymphocytes, monocytes and neutrophils, mediated by local presence of IL-6, IL-8 and TNF, are processes in which direct participation of RCEC could be relevant. These contributions of RCEC may have particular importance in renal allograft rejection, where these cells are the primary stimulus for leukocyte activation. However, also in other types of tubulo-interstitial disease including pyelonephritis, vasculitis and allergic interstitial nephritis, renal tubular epithelial cytokine release could be involved.

Documentations of primary epithelial cell cultures from a human organ expressing and secreting cytokines like IL-6, IL-8 or TNF are limited. IL-6 release has been shown by freshly isolated human dermal cells (48) and by human intestinal epithelial cells (49). IL-8 expression and release have been described in human retinal pigment epithelia (50). TNF production is, besides in some tumor cells (51, 52), very rare in non-hemo-

poietic cells. Our data, and recent data on RCEC secretion and expression of IL-8 (53) and TNF (22), thus extend the range of cell populations capable of IL-8 and TNF release. Additional evidence for a role for renal epithelial cells in producers of IL-6 comes from data on IL-6 release by rat mesangial cells (54-56) and by human renal cell carcinoma (57, 58).

The signal-transduction pathways which are involved in eliciting IL-6, IL-8 and TNF production are still unclear. For fibroblasts two independent pathways of IL-6 gene activation have been described; a protein kinase C-dependent and a cAMP-dependent signal transduction pathway (25). It seems clear that cytokine-release signal transduction pathways may vary depending on: A) the stimulus eliciting cytokine release (24, 59), B) the phenotype of the cell involved (59), and C) the cytokine which is released (60, 61). The latter finding is stressed by the clearly different induction profiles of IL-6 and TNF release by mononuclear phagocytes (23, 59, 62).

In parallel, we found that RCEC TNF-release differs from RCEC IL-6 and IL-8 release and from TNF-release by human mononuclear cells: Unstimulated RCEC show no TNF-release, but constitutively release high amounts of IL-6 and IL-8.

The concentrations of IL-1β and PMA needed for RCEC TNF-release were much higher than concentrations needed for RCEC IL-6 and IL-8 release, and for monocyte TNF release (23, 59, 63). TNF-release was, in contrast to IL-6 and IL-8, very heterogenous among RCEC from different donors; a finding which has also been described by Yard et al. (22). As for monocyte TNF-release, this inter-individual variatiqn was linked to stimulus-sensitivity rather then to the potential to release TNF after maximal activation (64).

Finally, LPS was, even at high concentrations, a very weak stimulus for RCEC TNF, IL-6 and IL-8 release. This might be related to the absence of surface-CD14 on RCEC, which is known to mediate activation of monocytes by low amounts of LPS (63, 65). In endothelial cells, which also lack CD14 expression, serum-derived soluble CD14 mediates LPS-activation (66, 67).

Why RCEC are less sensitive to LPS than endothelial cells, remains to be elucidated.

Since IL-1 induced RCEC TNF release, and stimulation with TNF triggers RCEC IL-6 and IL-8 release, TNF could be a second mediator for IL-1 induced IL-6 and IL-8 release. Addition of an excess of an inhibitory anti-TNF mAb did, however, not decrease IL-1 induced RCEC IL-6 and IL-8 release (data not shown), arguing against an important role for endogenous TNF release as a mediator for IL-1 induced RCEC IL-6 and IL-8 release. Furthermore, we investigated whether IL-6, which acts as an inhibitor of TNF-release in monocytes (23), has such a role in our model. Neither (pre)incubation with up to 1000 ng/ml IL-6, nor presence of an inhibitory anti IL-6 mAb, however, influenced IL-1 induced TNF release (data not shown). PGE_2 is another potential inhibitor of RCEC TNF release. PGE_2 is released by LPS and cytokine stimulated rat mesangial cells (68, 69), and reduces LPS induced TNF release by the same cells (69).

We measured, however, no clear influence of the cyclo-oxygenase pathway inhibitor indomethacin on unstimulated or IL-1 induced RCEC cytokine-release, suggesting no major

role for PGE_2 and other cyclo-oxygenase pathway products in our model. Further, we investigated whether budesonide, cyclosporin and FK 506, drugs which are used to prevent allograft rejection, reduce renal cytokine-release. Only the steroid budesonide inhibited cytokine release by RCEC and other cells. Budesonide induced reductions did, however, not exceed 50%, which is in contrast with the nearly complete steroid induced abrogation of TNF release by mononuclear phagocytes stimulated with endotoxin (41, 70). Incomplete inhibition of the release of neutrophil chemotactic activity by IL-1 stimulated renal epithelial cell cultures by corticosteroids has been reported previously (71). Whether these findings implicate principle differences between mononuclear phagocytes and renal epithelial cells in regard to their sensitivity to corticosteroids remains to be illucidated.

IL-6, IL-8 and TNF are multi-functional cytokines and important mediators of inflammatory processes. The capacity of RCEC to release these cytokines, as demonstrated in this study, argues for a model in which renal tubular epithelial cells are actively engaged in regulating inflammation. Which steps in the interaction between renal tubular cells and neighboring cells during inflammation finally lead to progressive tissue-damage and loss of renal function, appears to be a problem with increasing complexity.

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Chapter 3.3

ANTAGONISTIC EFFECTS OF LIPOPOLY-SACCHARIDE (LPS) BINDING PROTEIN AND BACTERICIDAL/PERMEABILITY-INCREASING PROTEIN ON LPS-INDUCED CYTOKINE RELEASE BY MONONUCLEAR PHAGOCYTES

COMPETITION FOR BINDING TO LPS

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Abstract

Serum proteins play an important role in lipopolysaccharide (LPS)-induced cell activation. The LPS binding protein (LBP) enhances cellular responses to LPS whereas the polymorphonuclear leucocyte product bactericidal/permeability-increasing protein (BPI) inhibits LPS-induced cell activation. In this study the influences of LBP and BPI, two proteins with opposite effects, but with considerable sequence homology, on LPS-induced mononuclear phagocytic cell cytokine release was studied. LBP was shown to enhance LPS-induced tumor necrosis factor (TNF)-a, interleukin (IL)-6 and IL-8 release by mononuclear phagocytic cells, whereas BPI inhibited the release of these cytokines. Furthermore, the effects of LBP and BPI on LPS-induced cytokine release by mononuclear phagocytic cells were shown to be counteractive. BPI interfered with the enhancing effect of LBP on the LPS-induced cytokine release. At high LBP to BPI ratios, BPI could no longer inhibit LBP induced enhancement. In accordance, increasing concentrations of BPI abrogated the LBP effect. Next it was shown that LBP and BPI compete for binding to LPS, by using an assay system that detects binding of free BPI to an anti-BPI monoclonal antibody (mAb). LPS prevented binding of BPI to anti BPI mAb, whereas preincubation of LPS with LBP prevented the LPS-induced inhibition. Also it was observed that both BPI and LBP inhibited LPS activity in the chromogenic LAL assay. We conclude from this study that LBP and BPI have counteractive effects upon LPS-induced mononuclear phagocytic cell cytokine release, by competing for binding to LPS.

Introduction

LPS is known to activate a number of cell types such as PMN, mononuclear phagocytes and endothelial cells. Serum proteins have been found to play an important role in LPS-mediated cell activation. Among these proteins is LPS binding protein (LBP), a 60 kDa acute phase reactant, which is also present in normal plasma (1, 2). This glycoprotein facilitates binding of LPS to CD14, a newly described receptor for LPS (3).

The presence of LBP increases the capacity of LPS 1) to prime PMN (4-6), 2) to induce cytokine release by mononuclear phagocytes (3, 7-9), and 3) to induce IgM expression by a CD14 transfected pre-B cell line 70Z/3 (10). In addition to LBP, a new group of serum proteins with LPS opsonizing activity for binding to CD14, called 'septin', has been described (11).

Also LPS inhibiting proteins have been reported. PMN produce the very potent bactericidal/permeability-increasing protein (BPI) which is stored in the azurophilic granule (12), but is also expressed on the cell surface (13, 14). This protein was shown to bind to LPS with high affinity (15-17), to induce bacterial killing (18, 19), and to inhibit effects of LPS in several in vitro settings. BPI inhibits LPS-induced PMN priming, TNF-α production in whole blood and activation of procoagulant proteases in Limulus amebocyte lysates (13, 20-22). BPI and LBP, two proteins with opposite effects upon LPS activation, have considerable sequence homology (1, 2, 23-25).

Furthermore, both bind to the lipid A part of LPS (7, 13, 20, 26, 27).

In this study, we investigated whether LBP and BPI affect, besides the LPS-induced TNF-α release, also the LPS-induced IL-6 and IL-8 release by mononuclear phagocytes. Furthermore we investigated the functional relationship between LBP and BPI on LPS-induced cytokine release. Our data indicate that the properties of LBP and BPI are counteractive.

These data prompted us to investigate whether these counteractive effects could be explained by competition for binding to LPS.

Materials and Methods

Reagents

Human rTNF-α was kindly provided by BASF/Knoll Ag (Ludwigshafen, FRG); human rIL-6 by Prof. W. Sebald, (Physiologisch-Chemisches Institut der Universität, Würzburg, FRG); human rIL-8 by Dr. I. Lindley (Sandoz Forschungsinstitut, Vienna); and human rIL-1β by Dr. S. Gillis (Immunex, Seattle, WA). LPS (from *Escherichia coli*, serotype 055:B5), the phorbol ester PMA and polymyxin B sulfate were purchased from Sigma (St. Louis, MO).

Macrophage serum-free medium (MØ-SFM) was obtained from GIBCO Europe (Paisley, Scotland). Human serum (HS) obtained from the local blood bank, was pooled, sterilized by a 0.2 µm filter, heated to 56°C for 30 minutes and stored at 4°C. HS contained <5 pg/ml endotoxin, as determined in the chromogenic LAL assay (Kabi Pharmacia, Mölndal, Sweden). Human rBPI was produced by transfected chinese hamster ovary cells and was purified sequentially by ion exchange column and by size exclusion column. Human rLBP was produced by the transfected human embryonic kidney cell line 293 EBNA provided by Invitrogen (San

Diego, CA). The conditioned medium contained ±2.5 µg/ml human rLBP. A BPI neutralizing mAb 4E3 was obtained by injecting mice with human rBPI followed by classical procedures. The selection and properties of the mAb will be discussed in detail elsewhere. In short, the antibody was selected on basis of the following properties 1) reactivity with human rBPI, 2) inhibition of the biological property of BPI to reduce the LPS-induced cell activation, as measured by mononuclear phagocytic cell TNF-α release, and 3) inhibition by LPS of mAb reactivity to BPI. An antiserum to BPI was obtained by immunizing rabbits with human rBPI. This anti-BPI antiserum did not cross-react with human rLBP as tested in ELISA assay.

Endotoxin determination

Endotoxin was measured using Chromogenic Limulus Amebocyte Lysate assay, performing the assay as described by the manufacturer (Kabi Pharmacia). For measurement of endotoxin content in serum, the serum was heated to 75°C for 5 minutes, to inactivate all serum proteins.

Cells

The monocytic cell line Mono Mac 6 was obtained from DSM (Braunschweig, FRG) (28). Human peripheral blood mononuclear phagocytes, obtained from buffy coats of healthy volunteers, kindly provided by the local blood bank, were isolated as described (29). In short, mononuclear cell suspensions obtained after Lymphoprep (Nycomed, Oslo, Norway) centrifugation were allowed to clump by low speed centrifugation at 4°C. Cell clumps, consisting for 80 to 95% of mononuclear phagocytes, were separated from the rest of the cells by sedimentation through ice cold HS. PMN contamination of the cell suspension was less than 5%.

Induction of cytokine-release

LPS, LBP and BPI were resuspended in MØ-SFM or in medium consisting of RPMI 1640 (GIBCO) and 10% HS. These reagents, alone or in combination were added in 96-well flat bottom tissue culture plates (Costar, Cambridge, MA) reaching an end volume of 150 µl/well, and incubated for 30 minutes at 37°C. Mononuclear phagocytic cells or Mono Mac 6 cells were washed five times to remove all serum-components, resuspended in the required medium and subsequently added to the plates (10^6 cells/ml mononuclear phagocytes or $4*10^6$ cells/ml Mono Mac 6, 50µl/well). Indicated concentrations of the reagents in the experiments refer to the concentrations at the beginning of the cell incubation, which were 75% of the pre-incubation concentrations. After 18 h incubation at 37°C, supernatants were harvested and kept at -20°C until use in the cytokine specific ELISA.

Competitive assay for LPS binding of LBP and BPI

Competition of LBP and BPI for binding to LPS was assayed with BPI specific mAb 4E3. This mAb recognizes only free BPI and does not interact with BPI that has formed a complex with LPS. The assay measures the influence of LBP on LPS-induced inhibition of binding of BPI to anti BPI mAb 4E3.

96-well immuno maxisorp plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 4E3. A concentration range of LPS was preincubated 30 minutes at 37°C, with different concentrations of LBP. Next, human rBPI (40 pg/ml) was added and the

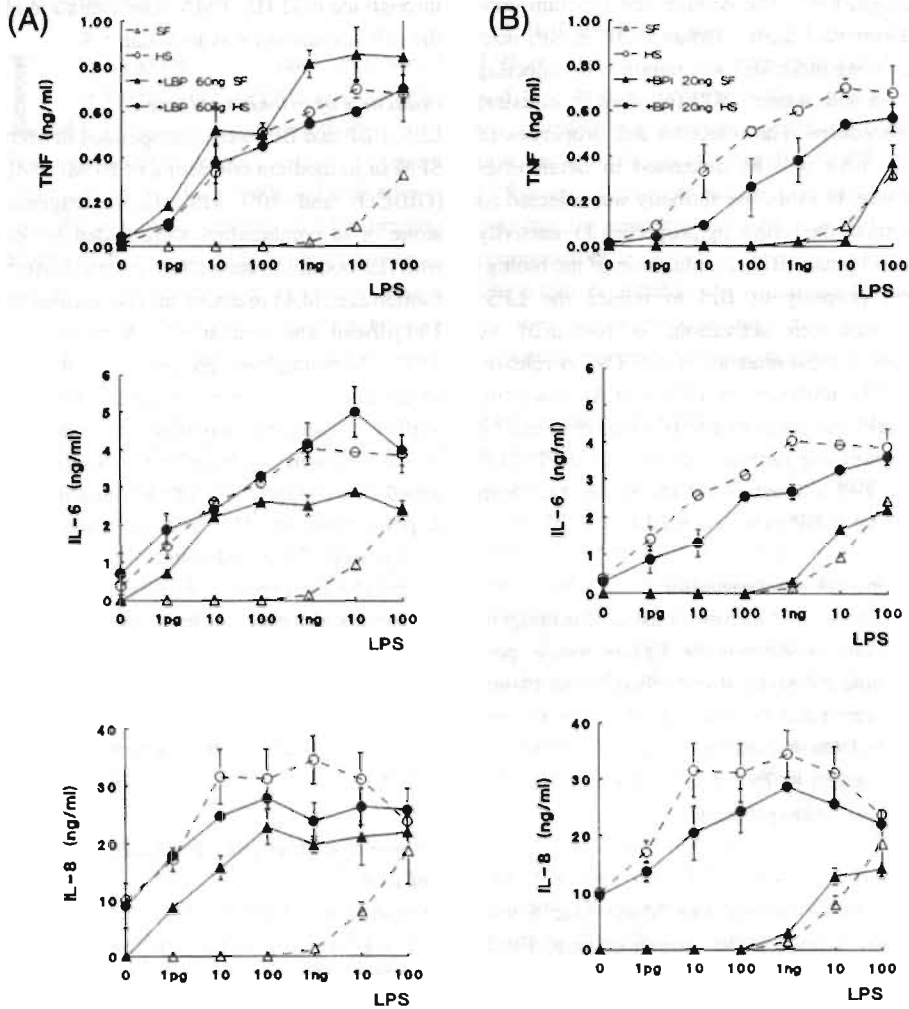

Figure 1. *Influences of LBP (A), and BPI (B) on LPS-induced mononuclear phagocytic cell cytokine release. A concentration range of LPS was preincubated 30 minutes 37°C with no other reagent, with 60 ng/ml LBP or with 20 ng/ml BPI, under serum-free conditions (SF) or in the presence of 10% HS. Mononuclear phagocytes were stimulated for 18 h and TNF-α, IL-6 and IL-8 release were determined in specific ELISA. Data are expressed as mean and SD of four measurements. SD bars are omitted if they fall within the symbol.*

mixture was further incubated 30 minutes at 37°C. Subsequently the mixture of reagents was added to the plates and incubated 1 h at room temperature. Washing and dilution of reagents were performed in assay buffer, consisting of 50 mM Tris, pH 7.4, 150 mM NaCl, 0.05% tween 20, 1 μg/ml polymyxin B sulfate. Assay buffer contained ±10 ng/ml

endotoxin. Plates were developed with polyclonal anti-BPI and peroxidase conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, Westgrove, PA). TMB (KPL, Gaithersburg MD) was used as a substrate for peroxidase. Photospectometry was performed at 450 nm.

TNF-α, IL-6 and IL-8 ELISA

Cytokine concentrations in the culture supernatants were determined using sandwich-ELISA for TNF-α (30), IL-6 (31) and IL-8 (32). In short, 96-well immuno maxisorp plates (Nunc) were coated overnight at 4°C with cytokine specific murine mAb. Human rTNF-α, human rIL-6 and human rIL-8 were used for standard titration curves. Test samples were added and incubated for 2 to 3 h at room temperature. Polyclonal rabbit anti-human TNF-α, anti-human IL-6 and anti-human IL-8 antiserum were followed by peroxidase conjugated goat anti-rabbit IgG (Jackson). TMB (KPL) was used as a substrate for peroxidase. Photospectometry was performed at 450 nm. The lower detection level of ELISA was 20 pg/ml for TNF-α, 10 pg/ml for IL-6 and 10 pg/ml for IL-8.

Results

Influences of LBP and BPI on LPS-induced mononuclear phagocytic cell cytokine release.

The influences of LBP and BPI on LPS-induced TNF-α, IL-6 and IL-8 release by mononuclear phagocytes were studied. To this end a concentration range of LPS was preincubated for 30 minutes at 37°C with 60 ng/ml LBP or 20 ng/ml BPI and added to mononuclear phagocytic cells either under serum-free conditions or in presence of 10% HS. Cytokine release by mononuclear phagocytic cells was shown to be strongly dependent upon presence of serum *(Fig. 1)*.

The enhancing effect of serum was less pronounced at higher LPS concentrations (10-100 ng/ml). Addition of LBP to different concentrations of LPS, in absence of serum, strongly enhanced mononuclear phagocytic cell TNF-α, IL-6 and IL-8 release, resulting in cytokine release comparable to that observed in presence of 10% HS *(Fig. 1A)*. In presence of serum, LBP did not influence LPS-induced cytokine release.

BPI inhibited mononuclear phagocytic cell TNF-α, IL-6 and IL-8 release partially in the LPS range from 1 pg to 10-100 ng/ml, depending on the cytokine studied, in presence of 10% HS *(Fig. 1B)*. Using higher BPI concentrations, up to 3 μg/ml, the inhibitory effect of BPI was enhanced, although never a complete inhibition was observed (data not shown).

Under serum-free conditions no inhibitory effect of BPI was observed. BPI did not reduce mononuclear phagocytic cell TNF-α-release in response to IL-1β and PMA in presence of serum (data not shown), indicating that BPI selectively reduced LPS-induced mononuclear phagocytic cell responses. Neither LBP nor BPI induced detectable amounts of mononuclear phagocytes cytokine release in absence of LPS *(Fig. 1)*.

A minor contamination of mononuclear phagocytic cell preparation with PMN could lead to PMN produced BPI, which may obscure the effect of exogenous BPI in the described serum-free experiments. Therefore the effect of BPI under serum-free conditions on the LPS-induced cytokine release by the

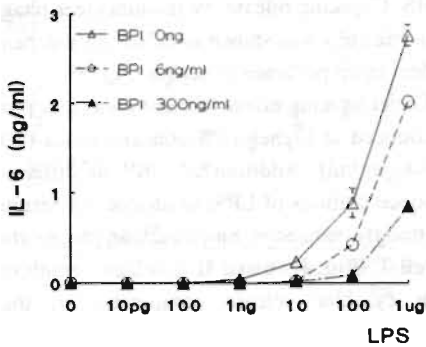

Figure 2. *Influence of BPI on LPS-induced IL-6 release by Mono Mac 6. A concentration range of LPS was pre-incubated 30 minutes 37°C with different concentrations of BPI under serum-free conditions. Cells were stimulated for 18 h and IL-6 release was determined in an ELISA. Data are expressed as mean and SD of four measurements. SD bars are omitted, if they fall within the symbol.*

monocytic cell line Mono Mac 6 was investigated. This cell line responds to relative high LPS concentrations by releasing cytokines (33). In these experiments, BPI substantially reduced LPS-induced IL-6 release *(Fig. 2)*. These data showed that BPI can, also in the absence of serum, inhibit LPS-mediated cell activation.

Counteractive effects of LBP and BPI on LPS activity

The observation that BPI and LBP have opposite effects upon LPS-induced cytokine release by mononuclear phagocytic cells and the reports that LBP and BPI, proteins with considerable sequence homology (25), both bind to lipid A part of LPS (26, 27) prompted us to investigate whether the effects of BPI and LBP are counteractive.

The influence of BPI on LBP-enhanced mononuclear phagocytic cell cytokine release by LPS was studied by pre-incubation (30 minutes at 37°C) a concentration range of LBP and LPS (1 ng/ml) in the presence or absence of BPI (3 ng/ml).

Fig. 3A shows that LBP, in a concentration dependent manner, enhanced LPS-induced TNF-α, IL-6 and IL-8 release. The presence of BPI reduced the enhancement of the response induced by LBP. BPI could no longer reduce LBP induced enhancement at a LBP concentration of 600 ng/ml.

Next, the influence of increasing concentrations of BPI on the enhancement by LBP (60 ng/ml) of the cellular responses to LPS (5 ng/ml) was studied.

In accordance with above mentioned data, BPI, even at higher concentrations, did not inhibit LPS-induced cytokine release by mononuclear phagocytic cells *(Fig. 3B)*.

However, BPI reduced the LBP enhanced cytokine release in a concentration dependent manner. At a BPI concentration of 3 μg/ml, there was no difference between LPS-induced cytokine release in the absence or presence of LBP. These results show that LBP and BPI have counteractive properties upon LPS-induced cell activation.

LBP and BPI compete for binding to LPS.

Next, it was investigated if the described counteractive effects of LBP and BPI upon LPS-induced cell activation were caused by competition for binding to LPS. To this end an assay was developed; competition of LBP and BPI for LPS binding was assayed by means of BPI specific mAb 4E3. This mAb, coated on the bottom of a 96-well plate, can only recognize free BPI and does not interact with BPI that has formed a complex with LPS. Binding of BPI to 4E3 was detected with polyclonal anti-BPI and peroxidase

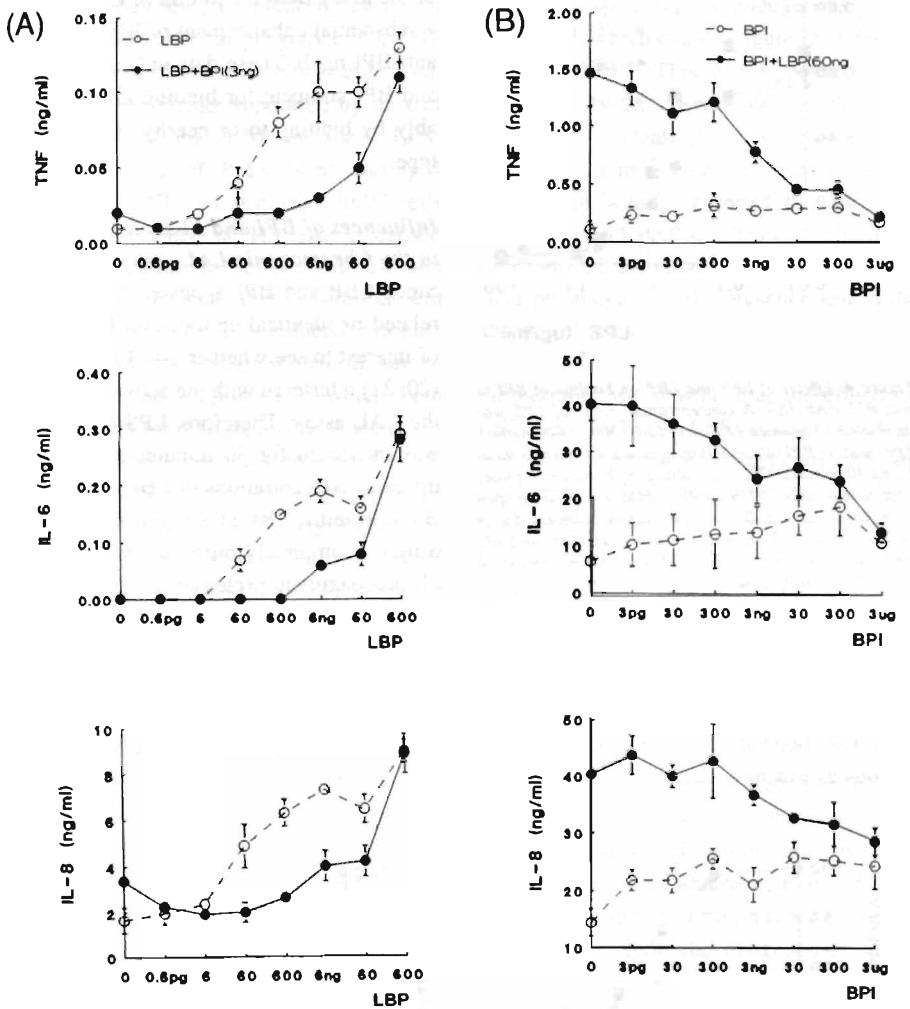

Figure 3. Antagonistic effects of LBP and BPI on LPS-induced mononuclear phagocytic cell cytokine release. Different concentrations of LBP were preincubated 30 minutes 37°C with 1 ng/ml LPS in presence or absence of 3 ng/ml BPI (A) or different concentrations of BPI were preincubated 30 minutes 37°C with 5 ng/ml LPS in presence or absence of 60 ng/ml LBP (B) under serum-free conditions. Cells were stimulated for 18 h and TNF-α, IL-6 and IL-8 release were determined in specific ELISA. Data are expressed as mean and SD of four measurements. SD bars are not shown, if they fall within the symbol.

conjugated goat anti-rabbit IgG.

The influence of LPS on BPI binding to the anti-BPI mAb 4E3 was measured in the presence or absence of LBP. The data showed that LPS prevented binding of BPI to 4E3 (Fig. 4). Addition of LBP abrogated the LPS-

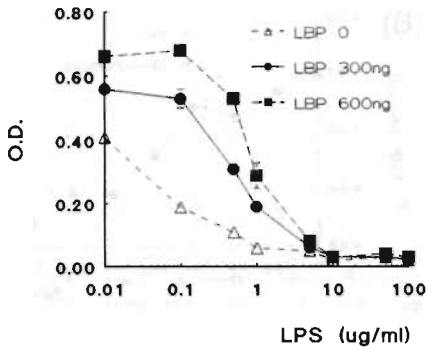

Figure 4. Effects of LPS and LBP on binding of BPI to anti-BPI mAb 4E3. A concentration range of LPS was incubated 30 minutes 37°C with LBP (300 or 600 ng/ml). After adding BPI (40 pg/ml) the mixture was further incubated 30 minutes 37°C and subsequently added to plates coated with anti-BPI mAb 4E3. Plates were developed with polyclonal anti-BPI and peroxidase conjugated goat anti-rabbit IgG. Data are expressed as O.D. and are mean and SD of three values. SD bars are not shown, if they fall within the symbol.

mediated inhibition of BPI binding. This effect appeared to be both LPS and LBP concentration dependent. Even at LPS concentrations as low as 10 ng/ml, the LPS content of the assay buffer, addition of LBP induced a substantial enhancement of BPI binding to anti-BPI mAb. These data indicate that LBP and BPI compete for binding to LPS, probably by binding to or nearby the same epitope.

Influences of BPI and LBP on LPS activity in the Chromogenic LAL assay

Since LBP and BPI appeared to react with related or identical epitopes on LPS, it was of interest to see whether also LBP, like BPI (20, 21) interfered with the activity of LPS in the LAL assay. Therefore, LPS (100 pg/ml) was incubated for 30 minutes at 37°C with different concentrations of LBP or BPI.

Both proteins, LBP at 1.5 µg/ml and BPI at 3 ng/ml completely prevented the activation of procoagulant proteases in the *Limulus*-amebocyte lysates by LPS *(Fig. 5)*.

Discussion

In this study we showed that LBP functions as a general enhancer of LPS-induced cell

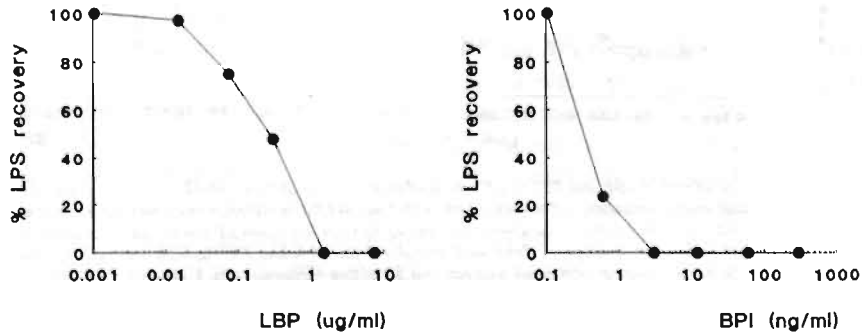

Figure 5. Influences of LBP and BPI on the activity of LPS in the chromogenic LAL-assay. LPS (100 pg/ml) was preincubated for 30 minutes at 37°C with different concentrations of LBP (A) or BPI (B). LPS-activity was then determined in the LAL assay. The effects of LBP and BPI are expressed as percentage of recovery of LPS activity in the LAL assay. One representative experiment out of a serie of three is shown.

activation. Besides LPS-induced release of TNF-a by mononuclear phagocytes (3, 7-9), also release of IL-6 and IL-8 was enhanced. These data are further supported by data of others showing that LBP enhances LPS-induced priming of PMN (4-6) and IgM expression by CD14 transfected 70Z/3 cells (10). The presence of HS also enhanced LPS-induced cytokine release strongly and addition of LBP under these conditions did not further enhance LPS-induced cytokine release. These data suggest that in human serum, LBP and septin (11), which both opsonize LPS for binding to CD14, are available in adequate amounts to opsonize all exogenous LPS. In serum a balance will exist between several LPS activity regulating proteins, among which soluble CD14 described to inhibit LPS activation (34, 35). Presence of these proteins could be the explanation for the observation that cytokine release in presence of serum-free medium supplemented with LBP is not exactly equal to cytokine release in presence of 10% HS.

BPI was shown to partially block, not only the LPS-induced release of TNF-α (13, 21, 22), but also the release of IL-6 and IL-8 by mononuclear phagocytic cells in presence of serum. In contrast, under serum-free conditions no effect of BPI was detected. The relatively high concentrations of LPS that are required to induce cytokine release under serum-free conditions might trigger a mechanism of LPS induced activation of mononuclear phagocytes, that is independent of CD14, LBP and BPI (31).
Another explanation for the lack of effect of BPI under serum-free conditions could be that a minor contamination with PMN of the mononuclear phagocytic cell suspension might be responsible for endogenously produced BPI, which could obscure the effect of exogenous BPI. This last hypothesis was confirmed by the observation that the LPS-induced IL-6 release, under serum-free conditions, of the monocytic cell line Mono Mac 6 was significantly inhibited by BPI. It is therefore concluded that BPI can inhibit the LPS-induced cytokine release by mononuclear phagocytes, in the presence and in the absence of serum.

Furthermore we showed that the effects of LBP and BPI on LPS-induced cytokine release by mononuclear phagocytic cells are counteractive. BPI, concentration dependently, interfered with the enhancing effect of LBP on the LPS-induced cytokine release. At high LBP to BPI ratios, BPI could no longer block the LBP-induced enhancement. In accordance, it was shown that increasing concentrations of BPI abrogated the LBP effect.

The antagonistic effects of LBP and BPI can explain the above discussed observation that BPI, in presence of serum, only partially inhibited the LPS-induced cytokine release. The LBP present in serum has an antagonistic influence upon the exogenously added BPI. Furthermore this phenomenon could explain the observations of Aida et al. that PMN are not able to inactivate LPS in presence of plasma (36). In line with the suggestion of the authors we hypothesize that LBP protects LPS against inactivation by BPI, produced by activated PMN.

Since both LBP and BPI are known to bind to the lipid A part of LPS (7, 13, 20, 26, 27), we investigated whether the antagonistic

effect was based on a competitive binding of LBP and BPI to LPS. Using an assay system that consisted of a detection system for free BPI, we are the first to show that BPI and LBP compete for binding to LPS. Moreover, the fact that both BPI (20, 21) and LBP inhibit LPS activity in the LAL assay provide further evidence for the theory that these two proteins bind to the same or near the same region on the LPS molecule. We therefore conclude that the observed antagonistic effects of LBP and BPI on LPS induced cytokine release by mononuclear phagocytic cells, can be explained by competition of LBP and BPI for binding to LPS.

Responses of cells to relatively low concentrations of LPS is mediated via the glycosylphosphatidylinositol anchored membrane protein CD14, which is strongly expressed on mononuclear phagocytes (37) and weakly on PMN (4). Interaction of LPS with CD14 is facilitated by LBP and by septin.
Here we showed that BPI may interfere in this signal transduction pathway by competing with LBP for binding to LPS and thus interfering with the LPS-LBP complex interaction with CD14. Both LBP and BPI were shown to be involved not only in LPS-induced TNF-α release (3, 7-9, 13, 21, 22), but also in LPS-induced IL-6 and IL-8 release, as was also recently described for CD14 (31). The fact that CD14 mediates LPS effects primarily at low LPS concentrations explains the observation that the effects of LBP and BPI upon LPS-induced cytokine release were absent at high LPS concentrations.

Since the observation that LBP and BPI have antagonistic effects upon LPS-induced cell activation we expect that the balance between these two proteins determines LPS activity under physiological conditions. The role of septin in this process needs to be elucidated. The nature of these proteins influencing LPS activity is quite different. BPI is rapidly produced by PMN in response to LPS and other stimuli, although only a small part is reported to be released (12, 13). LBP on the other hand is an acute phase reactant, which is continuously present in plasma, that increases with slower kinetics (2). Septin is also reported to be constitutively present in plasma (11).

Summarizing, in this study we investigated the functional relationship between LBP and BPI, two proteins involved in the response to gram-negative bacteria with considerable sequence homology, but with opposite effects. LBP and BPI were shown to have antagonistic effects upon the LPS-induced TNF-α, IL-6 and IL-8 release by mononuclear phagocytic cells, by competition for binding to LPS.

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Chapter 4.1

EVIDENCE FOR ENDOCYTOSIS OF E-SELECTIN IN HUMAN ENDOTHELIAL CELLS

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Summary

E-selectin is an inducible adhesion molecule on endothelial cells. The internalization of this glycoprotein was investigated on TNF-activated cultured human umbilical vein endothelial cells (HUVEC). Kinetics of inter cellular adhesion molecule-1 (ICAM-1) were studied in parallel-experiments. Internalization studies were performed with radioiodinated antibodies in an acid-elution endocytosis assay, and by immunohistology; both approaches gave equivalent results. [^{125}I]ENA1, a mAb specific for E-selectin, was internalized at a rate of approximately 1.7% of the membrane-bound [^{125}I]mAb per minute. In contrast, less than 0.1% of membrane bound [^{125}I]RR1/1, a mAb specific for ICAM-1, was internalized per minute. TNF-activated HUVEC were immuno-stained and examined by light-microscopy (LM) and electron microscopy (EM). LM revealed presence of ENA1, but not RR1/1, after 30 minutes of incubation with these mAb's in cytoplasmic vesicles, which were characterized as multivesicular bodies by EM. Without previous mAb-exposure of the endothelial cells, both high amounts of E-selectin and BSA complexed to colloidal gold, used as a marker for fluid phase internalization, were detected in the same organelles, thus arguing against mAb interaction induced E-selectin internalization. Furthermore, the amount of E-selectin surface expression was not influenced by ongoing mAb presence, also arguing against mAb interference with normal E-selectin kinetics.

Taken together, these results indicate that TNF-activated HUVEC constitutively internalize E-selectin. Physiological significance of E-selectin internalization in the regulation of E-selectin membrane-expression, and in clearing E-selectin ligands from the circulation, needs further investigation.

Introduction

E-selectin (consensus term for ELAM-1/LECAM2 (1)) is a 115kD membrane glycoprotein (2) which is transiently expressed on human endothelial cells after activation with TNF, IL-1 and bacterial LPS (2-5).

E-selectin is engaged in the adherence of neutrophil granulocytes (2 ,6), a subpopulation of monocytes (7, 8) and skin homing T-cells (9, 10) to activated endothelium.

In contrast with CD18 mediated adhesion, E-selectin offers a venous shear stress resistent, temperature insensitive anchoring (11, 12), for which neutrophil L-selectin (13) (consensus term for MEL14/LECAM1 (1)), and glycolipids and glycoproteins containing sialylated Lewis x tetrasaccharide-moieties (14-16) might function as a ligand. Neutrophil interaction with E-selectin does not play a role in activating neutrophil oxidative burst activity (17, 18) or in mediating neutrophil transmigration (13), although neutrophil CD11b/CD18 membrane expression and adhesive capacities are increased after interaction with E-selectin (19).

E-selectin is, together with the largely homologous adhesion molecules L-selectin and P-selectin, composed of an amino-terminal lectin-like domain followed by an epidermal growth factor-like domain and six repetitive motifs, related to those found in complement-binding proteins.

E-selectin has a trans-membrane domain and a short cytoplasmic tail (20). Biosynthetic labeling experiments performed by Bevilacqua et al. (20) and Smeets et al. [E.F. Smeets, submitted for publication] demonstrated that the E-selectin molecule has a short half-life, since labeled E-selectin molecules were largely lost 3 hours after labeling was terminated. Apparently, E-selectin molecules are not stably expressed on the cell-surface. Presence of a tyrosine residue in the intracellular tail of E-selectin has led to the hypothesis that E-selectin might be internalized via clathrin coated pits (20).

The present study was undertaken to investigate the kinetics of the E-selectin molecule. Using the E-selectin specific mAb ENA1 (6) as a marker, we demonstrated that E-selectin is constitutively internalized, and transferred to multivesicular bodies in the cytoplasm in TNF-stimulated HUVEC. ICAM-1 was investigated in parallel, and found not to be internalized.

Materials and Methods

Reagents and antibodies

Recombinant human TNF-a was kindly provided by BASF/Knoll AG. (Ludwigshafen FRG). mAb ENA1 (IgG1), reactive with E-selectin (6), was prepared as described (21). mAb RR1/1 (IgG1), a kind gift of Dr. Rothlein (Boehringer Ingelheim, Ridgefield, CT) is reactive with ICAM-1 (22). mAb MOPC21 (IgG1), a non specific control antibody, was kindly provided by Celltech (-Slough, UK).

Radioiodination of antibodies

Antibodies were radioiodinated using Iodobeads (Pierce, Rockford, IL) according to the manifacturer's instructions. Three Iodobeads were pre-loaded with 250 mCi $Na^{125}I$ (Amersham, Buckinghamshire, England) in 500 ml PBS for 5 minutes at RT. About 250 mg of protein G purified RR1/1 and ENA1 were added in a small volume and the incu-

bations were continued for 10 minutes. Subsequently, the iodobeads were removed and labelled protein and free iodine were separated on a Sephadex G-50 column (Pharmacia, Upsala, Sweden), equilibrated with 0.5% BSA (Sigma) in PBS. The radiolabeled protein (50-150 mCi/mg) was stored at -20°C.

Cells

HUVEC were obtained by collagenase treatment of the human umbilical vein. The cells were cultured in fibronectin-coated (fibronectin kindly provided by Dr. J. van Mourik, CLB, Amsterdam, The Netherlands) tissue culture flasks (Costar, Cambridge, MA) in RPMI1640 (Gibco Europe, Paisley, Scotland), supplemented with 10% heat-inactivated human serum, 10% heat-inactivated bovine calf serum (BCS) (Hyclone, Logan, UT), 50 mg/ml heparin (Sigma), 30 mg/ml endothelial growth supplement (Collaborative Research Incorporated, Bedford, MA) and antibiotics. Endothelial cells were characterized by their pavement-like monolayer morphology and positive staining with mAb hec7 directed against PECAM-1 (23), generously provided by Dr. W.A. Muller, Rockefeller University, NY.

Internalization assays

Two days before the experiment, endothelial cells of passage three were seeded on fibronectin coated 25 cm^2 tissue culture flasks. After activation with TNF (4-5 hours to study E-selectin, 12-18 hours to study ICAM-1), monolayers were rinsed twice with ice-cold PBS containing Ca^{2+}, Mg^{2+} and 0.1% BSA. Cell-surface antigens were labeled by 15 minutes of incubation with 10 mg/ml [^{125}I]mAb in PBS containing Ca^{2+}, Mg^{2+} and 0.1% BSA at 4°C. Excess of [^{125}I]mAb was removed and monolayers were rinsed extensively. Incubation of unstimulated HUVEC monolayers with 10 mg/ml [^{125}I] ENA1 bound less than 5% of the amount of ^{125}I bound to TNF-stimulated HUVEC. After labeling and washing, monolayers were incubated with pre-warmed (37°C) RPMI1640 10%BCS for various times. Subsequently, supernatant medium was collected and cells were rinsed two times with cold PBS containing Ca^{2+}, Mg^{2+} and 0.1% BSA. Supernatant medium and the latter two wash fractions were pooled and the amount of ^{125}I in this sample was measured in a gamma-counter. Next, monolayers were incubated for 10 minutes with cold 0.1 M citrate buffer of pH 3 containing Ca^{2+} and Mg^{2+}, rinsed once with the same buffer and twice with PBS containing Ca^{2+}, Mg^{2+} and 0.1% BSA, to remove cell-surface bound [^{125}I] mAb. Elution buffer fractions and wash fractions were pooled and the amount of ^{125}I in this sample was measured in a gamma-counter.

Cells were then solubilized by incubation with 1 M NaOH for 10 minutes at room temperature (RT). The amount of ^{125}I in the lysate was measured in a gamma-counter. The specific pH 3 insensitive fraction was calculated by correcting lysate-fraction measurements for the background amount of pH 3 insensitive cell-associated ^{125}I. Background pH 3 insensitive ^{125}I ranged from 5% to 9% of the total amount of cell-associated ^{125}I after labeling, and was defined as the amount of ^{125}I which remained cell-associated when cells where treated with pH 3 citrate buffer directly after labeling.

Immuno-staining

Two days before the experiment, endothelial cells of passage three were grown on micro-

scope slides coated with gelatin (Difco, Detroit, MI) fixed with 0.5% glutaraldehyde (Merck, Darmstadt, FRG) and covered with flexiperm chambers (Heraeus, Amsterdam, The Netherlands). After activation with 10 ng/ml TNF (5 hours to investigate E-selectin kinetics, 18 hours to investigate ICAM-1 kinetics), endothelial cell monolayers were rinsed twice with cold PBS containing Ca^{2+} and Mg^{2+} and, for membrane immuno-staining, fixed by a 5 minute incubation with 3% formaldehyde and 2% sucrose in PBS containing Ca^{2+} and Mg^{2+}, followed by extensive washing. For intracellular staining, permeabilization was performed by incubation with 96% methanol during 5 minutes at RT, rinsing, and incubation with aqua bidest, pH 7.4, containing 20 mM HEPES, 300 mM sucrose, 3 mM $MgCl_2$, 50 mM NaCl, and 0.5% Triton X-100 (Sigma), for 5 minutes at RT. Cells were then incubated with 5 mg/ml ENA1, RR1/1 or MOPC21 in RPMI1640 10% BCS at RT for 30 minutes, rinsed three times, incubated with horse radish peroxidase conjugated goat anti mouse antibodies in RPMI1640 10% BCS for 30 minutes at RT, and stained with 3-3'-Diaminobenzidine.4HCl (Sigma) plus 0.1% H_2O_2 and Meyer's Hematoxylin.

Electron microscopy

Two days before the experiment, endothelial cells of passage three were grown on fibronectin coated 75 cm^2 tissue culture flasks. Monolayers were preincubated as described in section 2.5. BSA-gold was prepared by conjugating BSA to colloidal gold particles of about 5 nm as described by Slot et al. (25), and added to part of the HUVEC monolayers during the last 30 minutes at 37°C. Monolayers were rinsed twice with ice-cold PBS containing Ca^{2+} and Mg^{2+}, and fixed with 0.1% glutaraldehyde and 1% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate buffer pH 7.4 for 2 hours. After three washings with PBS, cells were removed from the culture flask with a rubber policeman, spun down in 10% gelatin in PBS and stored overnight at 4°C in 1% formaldehyde in 0.1 M phosphate buffer. Ultrathin cryosectioning was done as described previously (26). Cells, which were preincubated with ENA1, were labeled on the cryosections with rabbit anti mouse IgG visualized with Protein A complexed to colloidal gold (25). Otherwise the cells were labeled on the cryosections with ENA1, rabbit anti mouse IgG and protein-A gold. The sections were examined in a Philips EM 201 C electron microscope operating at 80 KV.

Results

Kinetics of surface E-selectin and ICAM-1 using radioiodinated mAb as a marker.

Radioiodinated E-selectin specific mAb ENA1, and ICAM-1 specific mAb RR1/1, were used as markers for E-selectin and ICAM-1 respectively.

TNF-activated HUVEC monolayers were labeled at 4°C with [^{125}I] mAb, rinsed, and incubated at 37°C for various times to allow redistribution of E-selectin and ICAM-1. Supernatants were harvested to determine the amount of release of E-selectin and ICAM-1. Only small amounts of ^{125}I were measured in the supernatant of [^{125}I]ENA1 labeled HUVEC after incubation at 37°C for up to two hours *(Fig. 1A)*, indicating that E-selectin release and loss of surface-bound [^{125}I]ENA1 does not play a substantial role during the first two hours at 37°C after labeling. Up to 40% of the ^{125}I label was, however,

found in the supernatant of [^{125}I] RR1/1 labeled monolayers after 2 hours of incubation at 37°C, which could represent release of ICAM-1 (Fig. 1B). Direct evidence for release of both E-selectin and ICAM-1 from the cells, was acquired using ELISA's for soluble E-selectin and ICAM-1, and immunoprecipitation. These results are presented elsewhere (J.F.M. Leeuwenberg, submitted for publication).

To distinguish between internalized molecules and cell surface molecules, cells were then treated with a pH 3 elution-buffer. Cell lysates were prepared, and the relative distribution of [^{125}I]mAb in the elution buffer and in the cell lysate was determined.
After [^{125}I]ENA1 labeled cells were warmed to 37°C, an increasing amount of [^{125}I]ENA1 remained cell associated after elution *(Fig. 1)* and must therefore have been internalized into a compartment protected from the externally applied elution buffer *(Fig. 1A)*. From these data, an internalization rate of 1.7% of membrane E-selectin per minute was estimated by regression analysis *(Fig. 1A, inset)*, implicating a half-life of 40 minutes for a surface E-selectin molecule on 4 to 5 hours TNF-activated HUVEC. Similar experiments were performed in which 2 hour as well as 10 hour TNF-activated HUVEC were labelled with [^{125}I]ENA1. No clear surface E-selectin half-life differences were found between 2 hour, 4 hour and 10 hour

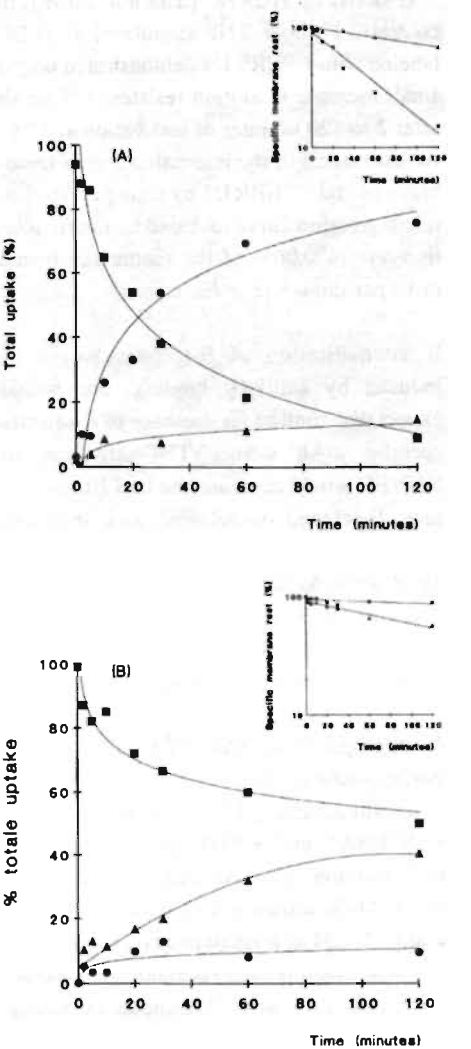

Figure 1: Redistribution of [^{125}I]ENA1 labelled E-selectin (A) and [^{125}I]RR1/1 labeled ICAM-1 (B) after different times at 37°C. HUVEC were activated with 10 ng/ml TNF (4 hours to study E-selectin, 12 hours to study ICAM-1) after which 10 µg/ml [^{125}I]mAb was added for 15 minutes at 4°C. Monolayers were extensively rinsed, incubated for the incubated time-interval at 37°C and samples were acquired as indicated in Materials and Methods. Supernatant fractions (▲), pH 3 sensitive membrane fractions (■) and the specific pH 3 insensitive endocytotic fractions (●) (corrected for non-specific [^{125}I]mAb presence measured at t=0), are represented as a proportion of the total amount of [^{125}I]mAb after labeling at 4°C. Inset: The relative reduction of the amount of membrane bound [^{125}I]mAb due to internalization only (●) and ^{125}I dissociation from the monolayer only (▲) after different times at 37°C, as calculated from the main figure data, and expressed in log scale.

TNF-activated HUVEC (data not shown). In contrast, 12 hour TNF-stimulated HUVEC labeled with [^{125}I]RR1/1 demonstrated only a small increase in elution resistent [^{125}I]mAb after 2 to 120 minutes of incubation at 37°C. An estimation of the internalization of membrane bound [^{125}I]RR1/1 by fitting a logarithmic regression curve revealed an internalization-rate of 0.06% of the membrane-bound mAb per minute *(Fig 1B, inset)*.

If internalization of E-selectin would be induced by antibody binding, one would expect that continuous presence of E-selectin specific mAb during TNF-activation of HUVEC would decrease the half-life of surface E-selectin molecules, and therefore reduce the amount of E-selectin surface expression. As shown in *Table 1*, no significant reduction in E-selectin expression was seen after 4 hours of activation, induced by either ENA1, RR1/1 or control IgG1 mAb.

Microscopic localization of E-selectin during endocytosis.

TNF stimulated HUVEC were incubated with ENA1 and RR1/1 at 37°C to allow redistribution of mAb-antigen complexes, and mAb-localization was evaluated by LM and EM. LM examination revealed visually unaltered membrane-expression of E-selectin and ICAM-1 after 30 minutes of incubation with respectively ENA1 and RR1/1 (not shown). After permeabilization of the cell

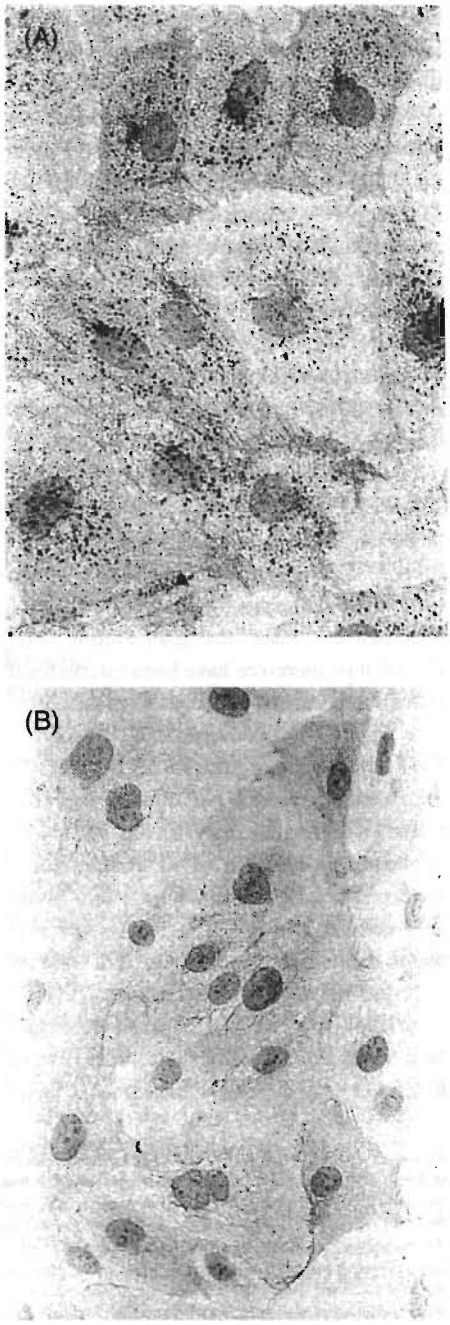

Figure 2: *Peroxidase-staining of 30 minute ENA1 (A) and RR1/1 (B) incubated HUVEC at 37°C. HUVEC was activated with 10 ng/ml TNF for 5 hours (for E-selectin staining) and 18 hours (for ICAM-1 staining). mAb were added during the last 30 minutes of TNF-incubation and immuno-staining of permeabilized cells was performed as mentioned in Materials and Methods. Heavily stained cytoplasmic vesicles are found in ENA1, but not in RR1/1 incubated monolayers.*

Preincubation	A_{492nm}
medium only	0.04 ± 0.01
TNF	1.41 ± 0.07
TNF + ENA1	1.31 ± 0.06*
TNF + RR1/1	1.45 ± 0.06
NF + MOPC21	1.44 ± 0.05

Table 1. Influence of mAb presence on E-selectin membrane expression.
HUVEC in microtiter-plates were incubated for 4 hours at 37°C in 200 µl/well RPMI1640 10% BCS and additives (10 ng/ml TNF and 10 µg/ml mAb as indicated). Plates were then rinsed three times with cold pH 3 citrate buffer, to remove cell surface antibodies. E-selectin expression was subsequently measured in ELISA using mAb ENA1 and peroxidase labeled goat anti mouse antibodies as described (21). Results indicate mean ± SD of 4 measurements.
** p=0.07, when compared to E-selectin expression in absence of mAb, as determined by the unpaired, two-tailed Student's t-test.*

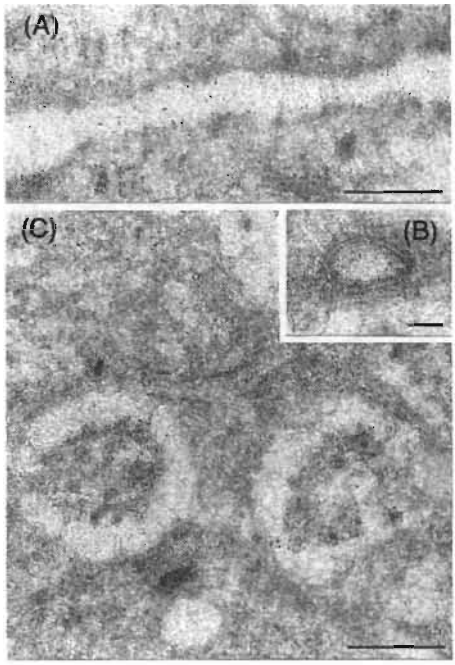

***Figure 3:** EM visualization of ENA1 in endo-lysosomal structures of 5 hour TNF activated HUVEC. mAb were added during the last 30 minutes of TNF-incubation. Cryosections were labeled with rabbit anti mouse IgG and then with protein-A gold (10 nm). Gold particles were found on the plasma membrane (A), in small vesicles close to the plasma-membrane (B), and in multivesicular bodies (C). Bars represent 0.5 µm (A and C) and 0.1 mm (B).*

membranes clear vesicles were seen in ENA1 incubated *(Fig. 2A)*, but not in RR1/1 *(Fig. 2B)* or control mAb (MOPC21, data not shown) incubated cells. Such vesicles were also found, using the anti E-selectin mAb ENA2 (21), 1.2B6 (27), and H4/18 (2) (data not shown), indicating that internalization did not involve mAb binding to a specific epitope on the E-selectin molecule. Monolayers incubated with ENA1 at 4°C did not show mAb containing vesicles (data not shown). EM examination of TNF-stimulated HUVEC, after incubation with ENA1 for 30 minutes at 37°C, revealed presence of this mAb in endo-lysosomal structures *(Fig. 3)*.

Gold particles were found on the plasma membrane *(Fig. 3A)*, in small vesicles close to the plasma-membrane (average diameter 0.15 mm. *Fig. 3B*), and in multivesicular bodies of bout 0.8 mm diameter *(Fig. 3C)*. Too few clathrin coated pits and clathrin coated vesicles were found in these sections, to allow conclusions on the involvement of the clathrin coated pit endocytotic pathway.

If E-selectin internalization occurred spontaneously, without being influenced by mAb-binding, internalized E-selectin should be present in the same endocytotic vesicles in TNF-activated endothelium without previous mAb incubation. Intra-cellular expression of E-selectin and ICAM-1 was therefore evalu-

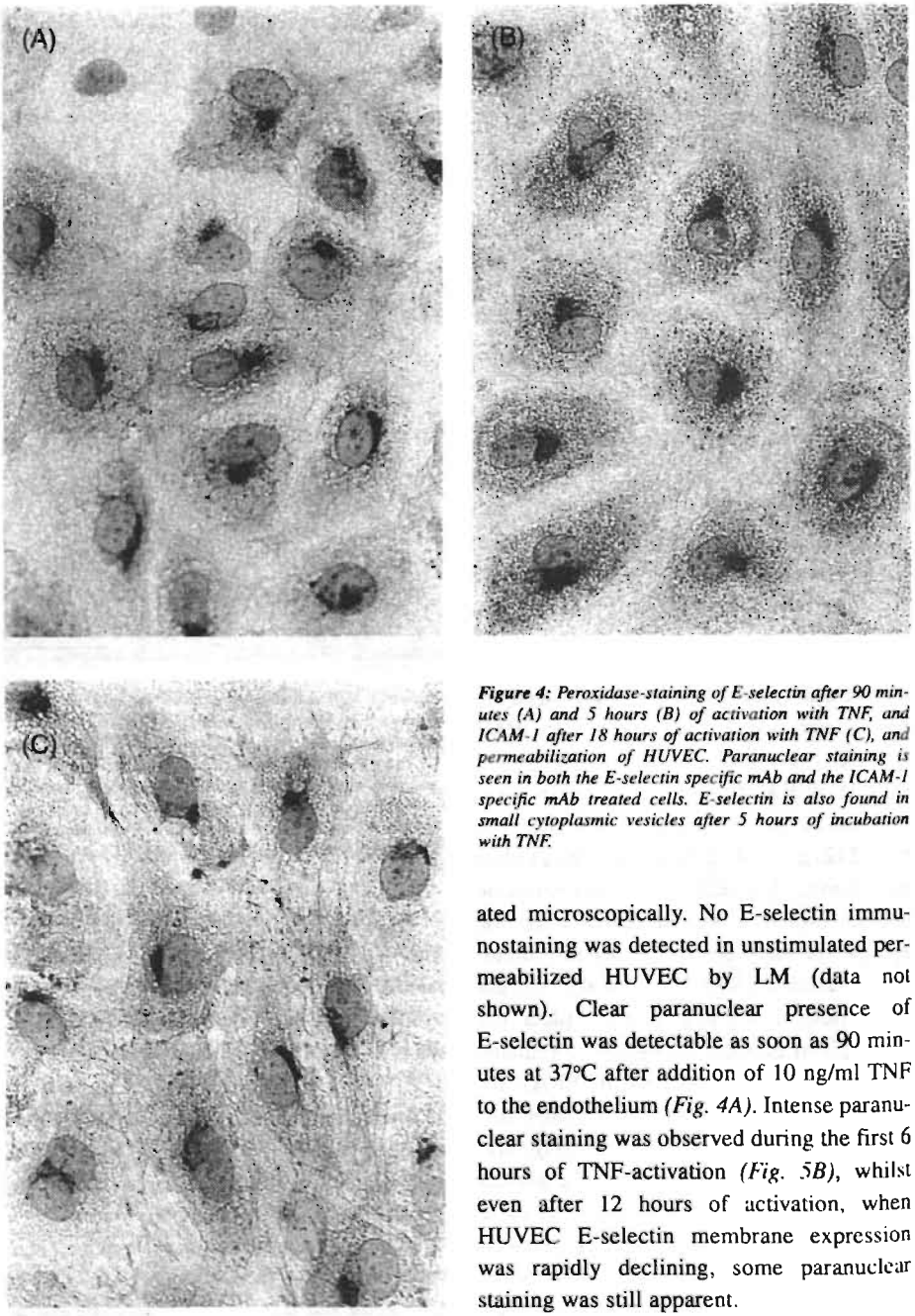

Figure 4: *Peroxidase-staining of E-selectin after 90 minutes (A) and 5 hours (B) of activation with TNF, and ICAM-1 after 18 hours of activation with TNF (C), and permeabilization of HUVEC. Paranuclear staining is seen in both the E-selectin specific mAb and the ICAM-1 specific mAb treated cells. E-selectin is also found in small cytoplasmic vesicles after 5 hours of incubation with TNF.*

ated microscopically. No E-selectin immunostaining was detected in unstimulated permeabilized HUVEC by LM (data not shown). Clear paranuclear presence of E-selectin was detectable as soon as 90 minutes at 37°C after addition of 10 ng/ml TNF to the endothelium *(Fig. 4A)*. Intense paranuclear staining was observed during the first 6 hours of TNF-activation *(Fig. 5B)*, whilst even after 12 hours of activation, when HUVEC E-selectin membrane expression was rapidly declining, some paranuclear staining was still apparent.

Five hours or more stimulated HUVEC showed a similar para-nuclear distribution pattern for ICAM-1 staining *(Fig. 4C)*. Cryosections of TNF-stimulated HUVEC were stained with ENA1, rabbit anti mouse IgG and protein A-gold. EM examination of these sections revealed the Golgi-apparatus as the origin of this para-nuclear E-selectin spot *(Fig. 5A)*. Besides in the Golgi-apparatus, LM also showed presence of E-selectin in cytoplasmic vesicles when HUVEC was stimulated with TNF for more than 3 hours *(Fig. 4B)*. These vesicles were characterized as multivesicular bodies by electron microscopy *(Fig. 5B)*, and showed the same characteristics as the previously described endolysosomal structures *(Fig. 3C)*. No ICAM-1 was detected in such organelles by either LM *(Fig. 4C)* or EM (data not shown).

If the ENA1 containing multivesicular bodies, stained after warming ENA1 labelled HUVEC to 37°C for 30 minutes would be identical to the spontaneously occurring E-selectin containing multivesicular bodies, E-selectin in the latter structures would originate from endocytosis of membrane E-selectin. To acquire direct evidence for the endocytotic content of these spontaneously occurring E-selectin containing multivesicular bodies, we added 5 nm BSA colloidal gold conjugates to HUVEC-medium during the last 30 minutes of a 5 hour TNF-activation interval, thus allowing internalization of these particles by fluid phase endocytosis. Cryosections were subsequently incubated with ENA1 and with rabbit anti mouse IgG and protein-A gold (10 nm). Endocytosed albumin-coated gold-particles were observed in multivesicular bodies, which were also labeled with anti E-selectin (Fig. 5C), demonstrating that these vesicles contain endocytosed material, which argues for cell-surface origin of E-selectin found in these spontaneously occurring multivesicular bodies.

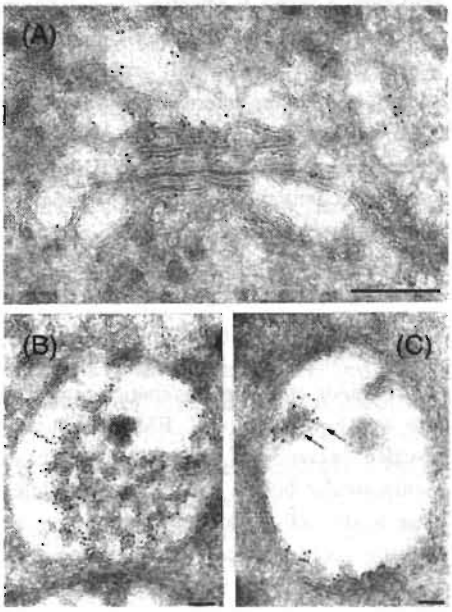

Figure 5: EM visualization of intra-cellular E-selectin antigen and co-localization of endocytosed BSA-gold conjugates in 5 hour TNF stimulated HUVEC. Cryosections were incubated first with anti E-selectin and then with rabbit anti mouse IgG and protein-A gold (10 nm). Gold particles were observed in the cisternae of the Golgi-apparatus, and most likely in the trans-Golgi network (A). Multivesicular bodies were heavily labeled (B). Part of the HUVEC monolayers were co-incubated the last 30 minutes at 37°C with BSA-gold (5 nm). The endocytosed albumin-coated gold-particles (arrows), were observed in multivesicular bodies, which were also labeled with anti E-selectin (large gold particles) (C). Bars indicate 0.5 μm (A) and 0.1 μm (B and C).

Discussion

We demonstrated in this study that surface E-selectin on TNF-activated HUVEC is rapidly internalized. Such internalization was demonstrated and quantified for mAb ENA1 labeled membrane E-selectin in an

acid-elution assay. E-selectin internalization was not caused by non-specific membrane internalization, since [^{125}I] mAb reactive with ICAM-1 were internalized at less then 0.1% per minute. Internalized mAb were stained and visualized by LM and EM. Intracellular accumulation of mAb was apparent after 5 minutes or more, when TNF-activated HUVEC cells were incubated with anti E-selectin mAb at 37°C, but was absent when the incubation was performed with anti ICAM-1 mAb and with isotype-matched control mAb, and when incubation-temperature was lowered to 4°C. EM examination revealed presence of internalized mAb in multivesicular bodies and in small vesicles close to the cell-membrane. The number of clathrin-coated pits and clathrin coated vesicles, found in the EM-sections, was too small to allow conclusions on the involvement of these structures in the internalization-process.

Direct study of endocytosis of surface-molecules requires some kind of surface-limited labeling or modification of the molecule. Such methods potentially interfere with normal molecule-traffic.

Therefore, indirect evidence was acquired suggesting that internalization of E-selectin occurred spontaneously instead of being induced by antibody-binding. E-selectin presence was detected without previous mAb exposure in vesicles apparently identical to the vesicles in which we found internalized ENA1, thus indicating that the localization of E-selectin in these multivesicular bodies also occurs in absence of mAb. Direct proof that these multivesicular bodies contained endocytosed material was obtained by demonstrating that fluid phase BSA-gold particles were co-internalized in these organelles. This argues strongly against other than endocytotic origin for E-selectin in these organelles. Additionally, no clear reduction in membrane-expression of E-selectin was induced by presence of an excess of ENA1, suggesting an unaltered half-life of surface E-selectin in presence of ENA1.

Furthermore, induction of internalization of E-selectin by antibody-binding would implicate some kind of signal transduction and cellular activation mechanism. We could, however, not detect any change in $[Ca^{++}]_i$.

Taken together, the presented results indicate that E-selectin is constitutively internalized, and that E-selectin bound antibodies are passively co-internalized. Since the cytoplasmic portion of E-selectin contains a tyrosine residue (20), which can be recognized by adaptor proteins mediating the formation of clathrin baskets, thus facilitating internalization in clathrin coated pits (28), clathrin coated pit mediated internalization appears to be the most likely mechanism for E-selectin internalization. No re-expression of [^{125}I]mAb labelled E-selectin after internalization was found. Instead, we measured an ongoing intracellular accumulation of ^{125}I in continuous presence of an excess of [^{125}I]ENA1 (data not shown).

These results are in line with the reported short half-life of biosynthetically labelled E-selectin molecules (20), and the ongoing presence of E-selectin in the Golgi apparatus of HUVEC after having reached maximal membrane expression (as demonstrated in this study), indicating ongoing protein synthesis.

Although the purpose of E-selectin internalization remains speculative, two possible functions emerge. Internalization prevents static presence of E-selectin molecules on

the cell surface, and thus enables endothelial cells to regulate the amount of surface-expression of E-selectin. E-selectin internalization might therefore be a crucial prerequisite for the rapid reduction in E-selectin membrane-expression, occurring after 4 to 6 hours of *in vitro* endothelial cell activation. The current identification the oligosaccharide moiety NeuNAca2-3Galß1-4(Fuca1-3)-GlcNAc, known generically as sialylated Lewis x (14-16), and of L-selectin (13) as potential E-selectin ligands, gave rise to the hypothesis that removal of E-selectin ligands from the circulation could be a major function of E-selectin internalization.

Most human cells do not express sialylated Lewis x. During transformation to malignancy, however, many adenocarcinoma-lines acquire the capacity to synthesize this oligosaccharide, leading to circulating mucin-like molecules with sialyl Lewis x moieties in patients with such malignancies (29, 30).

In parallel, another potential ligand of E-selectin, L-selectin (14), might be present in circulation during generalized infectious states, since rapid shedding of this receptor by neutrophils after activation with IL-8, FMLP or PMA has been reported in vitro (13, 31).

Both shedded L-selectin and soluble sialyl Lewis x moieties containing proteins can reduce neutrophil adherence to activated endothelium (16), and could therefore interfere with neutrophil recruitment or other neutrophil functions. Internalization of E-selectin might result in co-internalization of circulating sialyl Lewis x and shedded L-selectin, thus clearing these molecules from circulation, and re-establishing normal neutrophil-adherence. Generalized E-selectin expression during septic shock like syndromes (32, 33), and local E-selectin expression, found in the vasculature of human adenocarcinoma of the breast (own unpublished observation), could thus have a double influence on leukocyte adhesion.

First E-selectin offers a binding site for part of the leukocyte population, and secondly, E-selectin could decrease the amount of circulating adhesion inhibiting factors.

Besides a lectin-like domain, and an epidermal growth factor-like domain, involved in adherence of myeloid cells to endothelial cells (16), E-selectin contains six repetitive regions with sequence homology to known complement regulatory proteins (20). As suggested by Johnston et al. (34), these regions might bind C3b or C4b to facilitate clearance of circulating immune complexes. Seen from these viewpoints, endocytosis of E-selectin as described here, would be a mechanism critically linked to its function as a molecule involved in decontaminating the circulation in situations of severe immunological challenge.

In conclusion, this study demonstrates that E-selectin is internalized rapidly after having reached the cell-membrane. E-selectin internalization can occur together with an E-selectin bound antibody as a ligand, but is presumably not incited by this interaction. Further studies, to investigate the potential presence and function of co-internalization of physiological E-selectin ligands, might be of interest.

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Chapter 4.2

IFN-γ REGULATES THE EXPRESSION OF THE ADHESION MOLECULE ELAM-1 AND IL-6 PRODUCTION BY HUMAN ENDOTHELIAL CELLS IN VITRO

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Summary

In this study two new in vitro effects of IFN-γ on human umbilical vein endothelial (HUVE) cells were described. First, it was shown that the expression of the adhesion molecule el on activated HUVE cells can be modulated by IFN-γ. ELAM-1 is normally not expressed by HUVE cells, but its expression can rapidly be induced by TNF, IL-1, or LPS. Maximal expression is reached after 4 to 6 h of activation, and after 24 h the expression disappeared. whereas IFN-γ per se did not induce expression of ELAM-1, it enhanced and prolonged the expression of ELAM-1. this enhancement occurred when IFN-γ was added before or activation as well as when it was added simultaneously with activation. When IFN-γ was added 6 to 9 h after the activation, the normally ongoing reduction of expression was not only retarded, but the expression increased for at least 3 h. Moreover, IFN-γ abrogated the refractery period for restimulation. Neither IFN-β nor IL-6 had any effect on the expression of ELAM-1. The second effect of IFN-γ on HUVE cells is the capacity to enhance the IL-6 production by these cells. Prestimulation as well as coincubation of IFN-γ with TNF, IL-1 or LPS resulted in a strongly augmented production of IL-6. The effects of IFN-γ may in vivo play a role in the regulation of an inflammatory reaction, because ELAM-1 is an adhesion molecule for neutrophils, and IL-6 has an enhancing effect on the cytotoxicity of neutrophils.

Introduction

Infiltration of neutrophils into vessel wall is thought to be a hallmark in the development of inflammatory processes. This process starts with adhesion of neutrophils to endothelial cells. In this process cytokines such as IL-6 and neutrophil activating factor (IL-8) may serve as activators of neutrophils (1, 2).

IL-6 is an inflammatory cytokine with multiple effects including the ability to stimulate or enhance 1) the differentiation and proliferation of T cells (3), 2) the differentiation of B cells into plasma cells (4), 3) the production of acute phase proteins by hepatocytes (5), and 4) the toxicity of neutrophils (1).

Neutrophils as well as endothelial cells express surface molecules, that mediate adhesion. Inducible Ag on human endothelial cells have been described, which are normally not expressed on endothelial cells, such as ELAM-1 (6, 7), VCAM-1 (8), and the activation Ag described by Goerdt et al. (9). VCAM-1 is an adhesion molecule for lymphocytes (8), whereas ELAM-1 is involved in adhesion of neutrophils (6,7).

Knowledge of the regulation of the expression of the Ag may lead to a better understanding of the development of the inflammatory reactions. The inducers of ELAM-1, i.e., TNF, IL-1, and LPS, also induce the production of other cytokines such as IFN-γ, IFN-β, IL-6, and IL-8 (10-13).

The cytokines IFN-β, IFN-γ, and IL-6 are known to interact with endothelial cells and to interact with TNF, IL-1 and LPS, either synergistically or antagonistically (14-17). The influence on the induction and the expression of ELAM-1 and on the production of IL-6 was investigated. Whereas neither IFN-β nor IL-6 affected the ELAM-1 expression, IFN-γ enhanced the expression induced by TNF, IL-1 or LPS. Moreover, IFN-γ enhanced IL-6 production by activated endothelial cells.

Materials and Methods

Cells

HUVE cells cells were obtained by collagenase treatment of the human umbilical vein. The cells were cultured in fibronectin-coated (fibronectin kindly provided by Dr. van Mourik, CLB, Amsterdam, The Netherlands) tissue culture flasks (Costar, Cambridge, MA) in RPMI-1640 (Gibco Europe, Paisley, Scotland), supplemented with 10% pooled 0.2 μm filtered heat inactivated human serum (HS) derived from the local blood bank, 10% heat-inactivated bovine calf serum (BCS) (Hyclone, Logan, UT), 50 μg/ml heparin (Sigma), 30 μg/ml endothelial growth supplement (Collaborative Research Incorporated, Bedford, MA) and antibiotics. Endothelial cells were characterized by their pavement-like monolayer morphology, by positive staining with mAb hec7 directed against PECAM-1, generously provided by Dr. Muller (Rockefeller University, NY), and by positive staining with the anti-E-selectin mAb ENA1 after 4 hours of incubation with TNF.

Reagents and mAb

rHuman TNF-α was kindly provided by BASF/Knoll AG. (Ludwigshafen, FRG). rIL-1α was a kind gift of Dr. Gillis (Immunex, Seattle, WA). rIL-6 was a kind gift from Prof. Dr. W. Sebald (Wurzburg, FRG). rIFN-β was kindly provided by Prof. Bujard (Hoffman-La Roche, Basel, Switserland). rIFN-γ

was gift from Dr. P. van der Meide (TNO, Rijswijk, The Netherlands). LPS (phenol extract, chromatographically purified from Escherichia Coli, serotype 055:LB5) was purchased from Sigma (St. Louis, MO). Preparations of TNF, IL-1β and PMA were tested for endotoxin contamination by the chromogenic limulus amoebocyte lysate assay (Coatest, Kabi Diagnostica, Nyköping, Sweden), and were found to contain less than 5 pg/ml LPS in final solutions.

mAb ENA1 (IgG1) was obtained by immunizing mice with HUVE cells, pretreated with IL-1 and TNF during 5 h as described (18). mAb ENA1 is specific for endothelial cells activated with TNF, IL-1, LPS or phorbol esthers and is not reactive with other cell types. Cross-blocking studies with the labeled mAb ENA1 and 1.2B6 (kindly provided by Dr. D. Haskard, Devision of Medicie, UMDS, Guy's Hospital, London, UK), which is known to react with ELAM-1 (19), showed that mAb ENA1 and 1.2B6 reacted with the same epitope on activated HHUVE cells, being the ELAM-1 structure (data not shown). F(ab')2 fragments were prepared by pepsin digestion (Immobilized Pepsin, Pierce Chemical Co., Rockford, IL) as described (7). Complete digestion of the F(ab')2 preparation was ascertained by SDS-PAGE on Phast System separation unit (Pharmacia, Uppsala, Sweden).

Induction of Ag and cytokine release

HUVE cells cells of passage 3 were seeded at 10^4 cells/well in fibronectin-coated 96-well flat-bottom tissue culture plates (-Costar) in culture medium, one day prior to stimulation. HUVE cells were rinsed twice with RPMI-1640 and the medium was replaced by the appropriate agents in 200 µl/well RPMI-1640 supplemented with antibiotics, without growth factor, and with 10% HS (not heat-inactivated), or 0.1% BSA (endotoxin contamination <0.1 ng/g, Sigma) in experiments in which serum-free medium was employed. Media were tested for endotoxin contamination in the chromogenic limulus amoebocyte lysate assay, and were found to contain less than 5 pg/ml LPS. Supernatants were harvested and kept at -20°C until use in the IL-6 ELISA. Endothelial cell monolayers were washed, fixed with 0.05% glutaraldehyde for 10 minutes at room temperature, and kept at 4°C until use in the ELAM-1 ELISA.

IL-6 ELISA

The IL-6 concentration in the culture supernatant was determined using a sandwich-ELISA for IL-6. In short, 96-well immuno maxisorp plates (Nunc, Roskilde, Denmark) were coated with mAb CLB.IL6-8 (kindly provided by Dr. L. Aarden, CLB, Amsterdam, The Netherlands) directed against human IL-6 (20). rHuman IL-6 (a generous gift from Prof. Dr. Sebald, Physiologisch-Chemisches Institut der Universität, Würzberg, FRG) was used for standard titration curves. Test samples were added. Plates were incubated with polyclonal rabbit anti-human IL-6 antibodies, followed by peroxidase conjugated goat anti-rabbit IgG (Jackson, Westgrove, PA). O-phenylene-diamine (Sigma) was added as a substrate and photospectometry was performed at 492 nm. Standard titration curves were not influenced by presence of serum in the sample buffer. The IL-6 ELISA had a lower detection limit of 10 pg/ml IL-6.

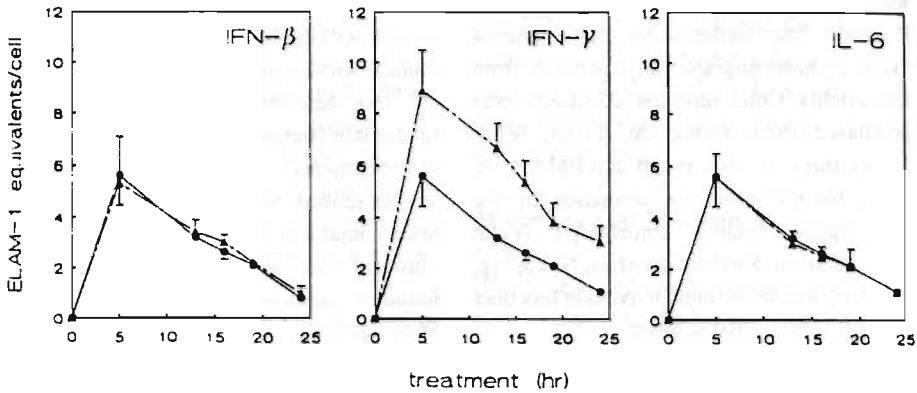

Figure 1. Effects of rIFN-β, rIFN-γ, and rIL-6 on the expression of ELAM-1 on HUVE cells. HUVE cells were activated with 10 U/ml IL-1 in the absence (●) or presence (▲) of rIFN-β (450 U/ml), rIFN-γ (100 U/ml) or with IL-6 (20,000 U/ml). At the different intervals ELAM-1 expression was determined in ELISA, followed by crystal violet staining of cells. ELAM-1 expression was given in mean ±SD of ELAM-1 equivalents per cell as described in Materials and Methods.

Detection of ELAM-1 expression

ELAM-1 expression was determined by ELISA using the ELAM-1 specific mAb ENA1, followed by peroxidase conjugated goat anti-mouse IgG. O-phenylene-diamine (Sigma) was added as a substrate and photospectometry was performed at 492 nm.

To correct for differences in cell numbers between individual wells, we performed a standard crystal violet staining, after the ELISA in the same wells. OD values were adjusted for the amount of crystal violet staining after substraction of the background, and for the ELISA-specific OD curve, and referred to ELAM-1 equivalents per cell.

Results

Effect of rIFN-β, rIFN-γ, and rIL-6 on induction and expression of ELAM-1

To investigate whether the cytokines rIFN-β, rIFN-γ, or IL-6 interfere with the expression of ELAM-1, we studied 1) the effect of simultaneous addition of inducer and rIFN-β, rIFN-γ, or rIL-6, and 2) the effect of preincubation with rIFN-β, rIFN-γ, and rIL-6 on ELAM-1 expression.

First the effect of simultaneous incubation of rIFN-β, rIFN-γ, or rIL-6 and an inducer of ELAM-1 was studied. In *Figure 1* the results are shown of a representative experiment using rIL-1 as an inducer of ELAM-1 expression at a concentration of 10U/ml. Neutrher rIFN-β nor rIL-6 affected the kinetics of ELAM-1 expression. rIFN-γ had a significant enhancing effect on ELAM-1 expression. The peak expression was enhanced, and it was shown that the expression decreased to a lesser degree. In *Figure 2* the results are given of an expirement with different inducers of ELAM-1 at a range of concentrations and with rIFN-γ as a costimulator at a concentration of 30 and 100 U/ml. At all concentrations of inducer, even when optimal concentrations of inducer were used, 10 ng/ml rTNF, an enhancing effect of IFN-γ

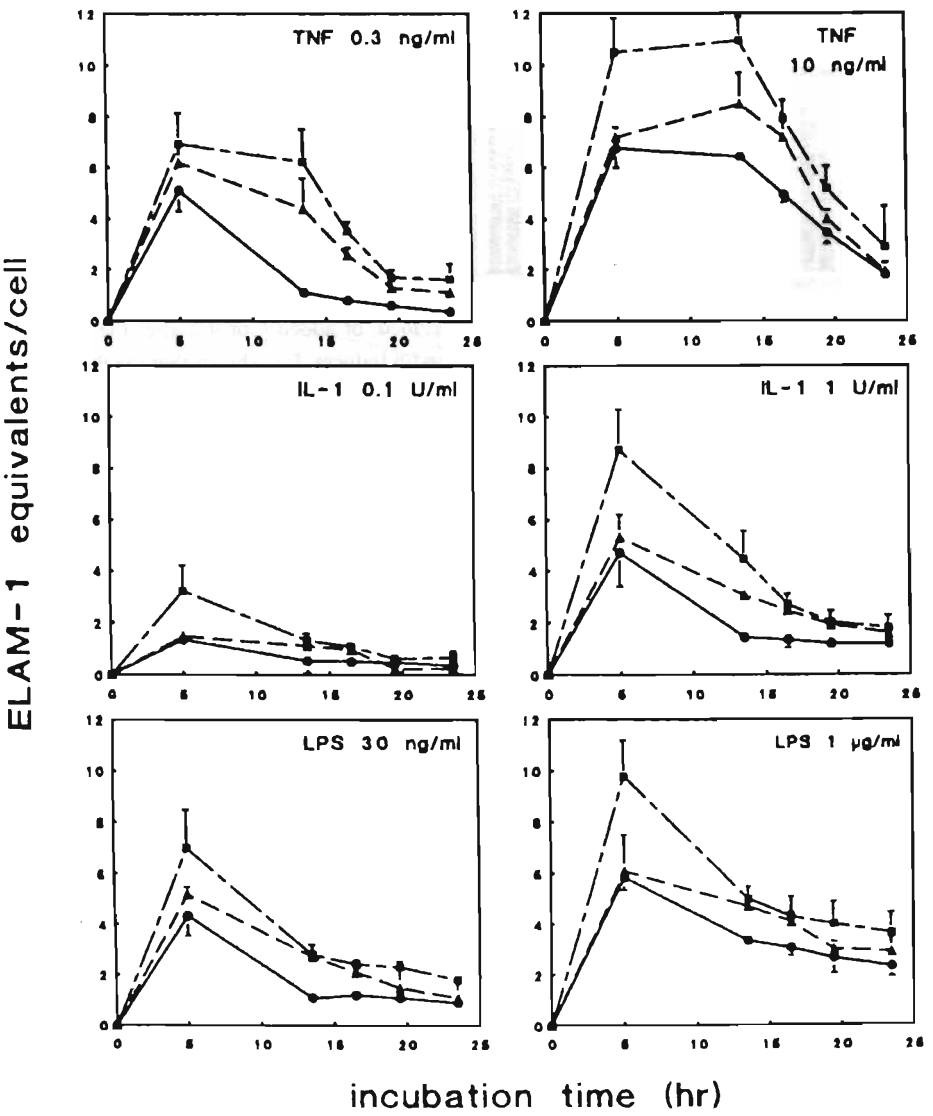

Figure 2. Effects of rIFN-γ (not added ●, 30 U/ml ▲, and 100 U/ml ■) on the expression of ELAM-1 by HUVE cells incubated wit rTNF, rIL-1, or LPS in the concentrations indicated. At the different intervals ELAM-1 expression was determined in ELISA. ELAM-1 expression was given in mean +-SD of ELAM-1 equivalents per cell as described in Materials and Methods.

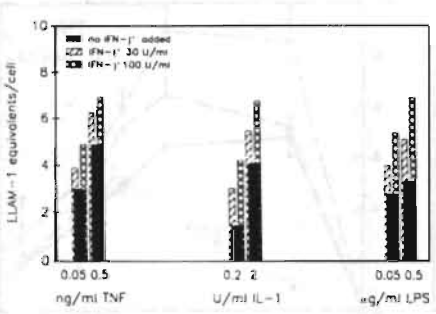

Figure 3. *Effects of preincubation with rIFN-γ on ELAM-1 expression. rIFN-γ (30 and 100 U/ml) was added 20 h before activation of the HUVE cells with rTNF, rIL-1, or LPS. After a next 5 h ELAM-1 expression was determined in ELISA. ELAM-1 expression was given in mean ELAM-1 equivalents per cell as described in* ***Materials and Methods****. SD of quadruplicates were less than 10% of the mean (not shown).*

on the ELAM-1 expression was observed. The heigt of the maximum expression was not only enhanced, but rIFN-γ also broadened the peak of expression.

Next the effect of pretreatment with rIFN-β, rIFN-γ, rIL-6 on ELAM-1 was studied. This was performed by preincubation of HUVE cells during 20 h with rIFN-β, rIFN-γ, or rIL-6. The cells were subsequently washed and incubated with different concentrations of rTNF, rIL-1, or LPS.

After 5 h the ELAM-1 expression was determined. Pretreatment of the HUVE cells with rIFN-β (up to 450 U/ml) or rIL-6 (up to 20,000 U/ml) had no effect on the expression of ELAM-1 induced by the different concentrations of rTNF, rIL-1, or LPS (data not shown). Pretreatment of the cells with rIFN-γ, however, caused an enhancement of the ELAM-1 expression as shown in *Figure 3*.

Effect of rIFN-γ on ELAM-1 expression when added after activation

Next we investigated the effect of rIFN-γ on HUVE cells already activated with rTNF or rIL-1. In *Figure 4* the results are shown of two seperate experiments, in which HUVE cellswere activated with 2 ng/ml rTNF or with 10 U/ml IL-1 as inducer of ELAM-1 expression.

rIFN-γ was either omitted, or simultaneously added, or added 6 or 9 h after the activation with inducer. It is shown that the down-regulation of the expression is interrupted; rIFN-γ up-regulates the expression in contrast to the ongoing reduction in the control cultures.

table 1

effect of IFN-γ on refractory period

second activation[b]	prestimulation[a]		
		LPS	LPS+IFN-γ
	0.052[c]	0.048	0.261
TNF(ng/ml)			
0.01	0.213	0.165	0.491
0.1	1.423	1.168	1.456
LPS(ng/ml)			
50	1.280	0.307	1.062
500	1.720	1.221	1.642

[a] *HUVE cells were prestimulated or not with LPS (1 μg/ml), or with LPS (1μg/ml) combined with IFN-γ (100 U/ml) during 20 h.*

[b] *HUVE cells were reactivated with TNF or LPS during 5 h at the indicatedconcentrations.*

[c] *ELAM-1 expression was determined in ELISA 5 h after the second activation.The data were reported as the mean absorbance units from quadruplicate determinations after substraction of the background. SD of quadruplicates were less than 10% of the mean (not shown).*

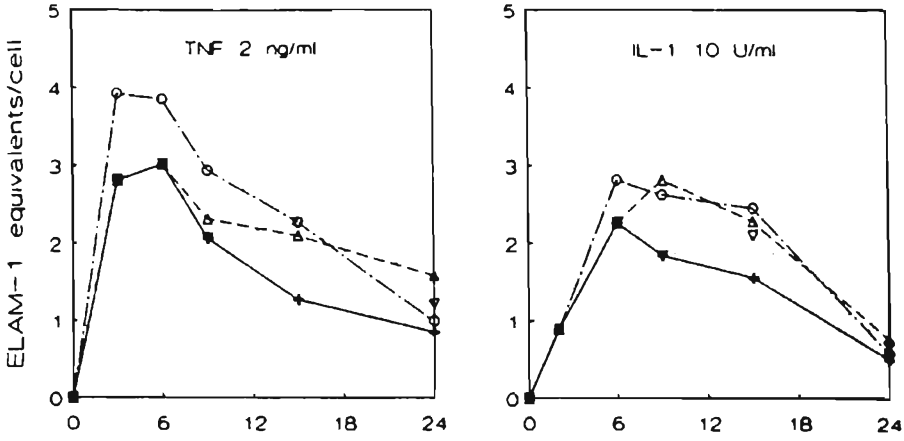

Figure 4. Effects of rIFN-γ (100 U/ml) on the expression of ELAM-1 by HUVE cells incubated wit rTNF or by rIL-1 (+), when added simultaneously (O) with rTNF or rIL-1, or added 6 h (Δ), or added 9 h (∇) after activation with rTNF or rIL-1. At the different intervals ELAM-1 expression was determined in ELISA. ELAM-1 expression was given as mean ELAM-1 equivalents per cell as described in ***Materials and Methods****. SD of quadruplicates were less than 10% of the mean (not shown).*

Effect of rIFN-γ on refractory period of ELAM-1 induction.

A refractory period was described for the induction of ELAM-1, i.e., cells activated with inducer, e.g., TNF, become refractory to restimulation by the same inducer TNF, but to a lesser degree by another inducer, e.g., rIL-1 or LPS (21).

In *Table 1* the results are shown of an experiment in which the effect of rIFN-γ on the refractoriness was investigated. HUVE cells were prestimulated with LPS, in the presence or absence of 100 U/ml rIFN-γ. After 18 h the cells were washed and restimulated with rTNF and LPS at two concentrations each. ELAM-1 expression was determinde 5 h after the restimulation.

Prestimulation with LPS did not completely abolish the signal, induced by the second stimulation. The effect of restimulation by rTNF as well as by LPS was reduced and this effect was most pronounced usinf the lowest concentration of LPS. Prestimulation, however, in the presence of rIFN-γ abrogated largely the refractoriness for restimulation as demonstrated by an enhanced ELAM-1 expression.

Effect of rIFN-γ on IL-6 production by HUVE cells.

Having established a role of IFN-γ on the expression of the adhesion molecule ELAM-1, we investigated the effect of rIFN-γ on the production of the inflammatory cytokine IL-6. At the same intervals at which ELAM-1 expression was measured in the experiments mentioned above, samples of the HUVE cells cultures were taken to measure the IL-6 content. Firs, we looked at the effect of rIFN-γ in the above mentioned

table 2

IL-6 production of HUVE cells 13 and 20 h after incubation with a combination of ELAM-1 inducer and IFN-γ

ELAM-1 inducer	13 h			20 h		
		30 U[a]	100 U		30 U	100 U
	40[b]	240	320	60	150	180
TNF (ng/ml)						
0.3	130	470	480	300	550	560
10	480	750	760	550	1830	1950
IL-1(U/ml)						
1	300	410	620	1.110	1.880	2.040
10	3.060	6.310	7.850	4.510	18.090	20.000
LPS (μg/ml)						
0.03	560	730	750	690	990	1.160
1	1.220	1.700	1.750	2.040	2.470	3.310

[a] *U/ml IFN-γ added.*
[b] *Means of IL-6 production in U/ml of quadruplicate wells; SD were less than 10% of the mean (not given).*

costimulation experiment, in which IL-6 production was measured 5, 14, 17, 20 and 24 h after stimulation of the HUVE cells.

As shown in *Figure 5*, rTNF, rIL-1, and LPS induced IL-6 production, and this production was strongly increased by the addition of rIFN-γ. In *Table 2*, the results are shown of the IL-6 production after 13 and 20 h of simultaneous stimulation with different concentrations of rTNF, rIL-1, or LPS, and 30 or 100 U/ml of rIFN-γ. rIFN-γ had an overall enhancing effect on the IL-6 production.

This effect was most pronounced in the combination with 10 U/ml IL-1, when 100 U/ml rIFN-γ caused a six-fold increase of IL-6 production. Although variability exists in the different experiments, the enhancing effect of rIFN-γ on IL-6 production was observed in four seperate experiments.

Effect of preincubation of HUVE cells with rIFN-γ on IL-6 production.

The effects of prestimulation with rIFN-γ on the IL-6 production by HUVE cells are shown in Figure 6. HUVE cells were preincubated during 20 h with rIFN-γ, and subsequently washed before activation with rTNF, rIL-1, or LPS. Five h after activation IL-67 production was measured in ELISA. In these excperiments, rIFN-γ, rTNF, rIL-1, LPS and

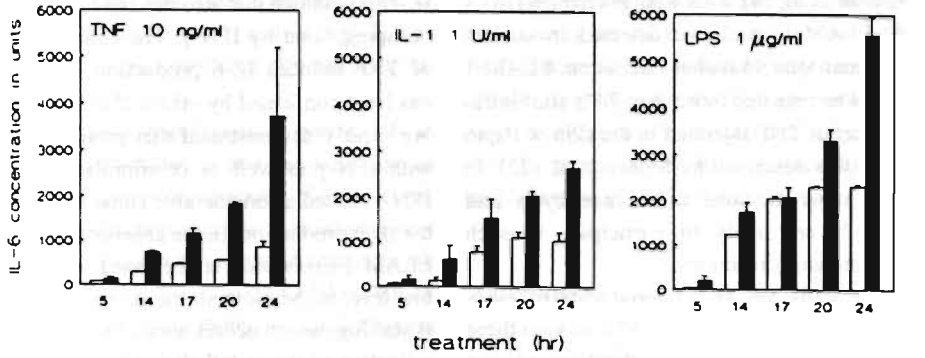

Figure 5. Production of IL-6 by HUVE cells, stimulated with rTNF (10 ng/ml), rIL-1 (1 U/ml), LPS (1 µg/ml) (open bars), or with a combination (closed bars) of rIFN-γ (100 U/ml) and forementioned activators.
Samples were taken at the different intervals indivcated to determine IL-6 production. Mean ± SD of quadruplicate wells are given.

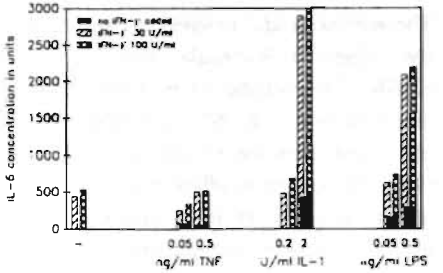

Figure 6. Effect of rIFN-γ (100 U/ml) on production of IL-6 by HUVE cells, when added 20 h before stimulation with rTNF, rIL-1, or LPS.
Samples were taken after a next 5 h for IL-6 measurement. Means of quadruplicate wells are given: SD of quadruplicate wells never exceeded 10% of the mean (not shown).

the combination ofrIFN-γ and rTNF induced only a weakly IL-6 production (<600 U/ml). A strong potentiating effect, however, of IL-6 production was observed, when HUVE cells were preincubated with rIFN-γ and subsequently activated with 2 U/ml rIL-1 or with 0.5 µg/ml LPS leading to an IL-6 production of 3000 U/ml within 5 h.

Discussion

In this study the effect of the cytokines IFN-γ, IFN-βm, and IL-6 on IL-1, TNF, and LPS-activated human endothelial cells was explored. The activation of endothelial cells by the mediators IL-1, TNF, and LPS was shown by the rapid induction of an adhesion molecule ELAM-1, and by the production of IL-6.

The kinetics of the expression in vitro of ELAM-1 are well known; maximal expression of this Ag occurs after 4 to 6 h of stimulation with TNF, IL-1, LPS or phorbol esters, after which the expression decliones despite the contineous presence of activator (18,21).

Several in vivo dat, however, showed an expression of activation Ag for a longer peri-

od, e.g., the activation antigen described by Goerdt et al. (9) with similar characteristics as ELAM-1, could be detected in contact eczemas after 96 h after elicitation. ELAM-1 could be detected more than 24 h after intracutaneous TNF injection in the skin of Papio anubis as described by Munro et al. (22). In vivo other immune cells, monocytes and T cells are likely to participate in such inflammatory reactions.

These cells are known producers of cytokines and it is conceivable that in vivo these cytokines modulate the duration of the expression of this adhesion molecule. It was found, that IFN-γ, but not IFN-β or IL-6 enhanced the ELAM-1 expression and retarded the decrease. Doukas and Pober (23) reported recently a similar effect using the combination of TNF and IFN-γ. Moreover, IFN-γ abrogated the refractoryness to restimulation. The mechanism via which ELAM-1 Ag expression decreases, is not yet understood.

The decrease of the expression after 4 to 6 h could be provoked by an unknown soluble factor released by the endothelial cells in the culture medium. Supernatants, however, derived from endothelial cells activated with TNF, IL-1,or LPS have no decreasinf effect on ELAM-1 expression (J. F. M. Leeuwenberg, unpublished observation).

These findings suggest, that the down-regulation of ELAM-1 is an intracellular event. IFN-γ is apparently able to interfere with this process and this effect may partly explain the in vivo data.

Further evidence for the activated state of endothelium is provided by the production of IL-6. Knowledge on the role, which IL-6 may play in a local inflammatory process is limited. Endothelial cells are known producers of IL-6, when activated by TNF, LPS, and IL-1. It is shown that also this production can be upregulated by IFN-γ. The enhancement of TNF induced IL-6 production by IFN-γ has been confirmed by others (24).

We clearly demonstrated that preincubation with IFN-γ as well as co-stimulation with IFN-γ caused a conciderable enhancement of the IL-6 production. In the absence of IFN-γ, ELAM-1 expression is decreased to reach a low level at the moment the IL-6 production is starting, which occurs about 5 to 10 h after activation of the endothelial cells.

The regulatory role of IFN-γ as coordinator of the inflammatory reaction seems evident. The enhanced and prolonged expression of the adhesion molecule for neutrophils ELAM-1, in conjunction with the accelerated production of IL-6, which stimulates the neutrophil merdiated cellular cytotoxicity (1), contribute to an efficient progress in the inflammatory reaction. Furthermore, IL-6 may accelerate the inflammatory reaction by stimulating T and B cells, leading to further IFN-γ production.

A number of adhesion molecules are known, which expression is known to be regulated by cytokines. In addition to ELAM-1, an adhesion molecule VCAM-1, inducible by TNF and IL-1 on endothelial cells has recently been described (8). VCAM-1 is an adhesion molecule for lymphocytes and is thought to play a role in acute as well as in a chronic inflammation, whereas ELAM-1 is involved in the acute inflammatory reaction. Whether IFN-γ also medulates the expression of VCAM-1 as shown for ELAM-1, remains to be investigated.

The expression of the adhesion molecule ICAM-1, but so far known not ICAM-2 (25),

can be up-regulated by TNF, Il-1, and IFN-γ on many cell types (26). The expression of the ligand of ICAM-1 and ICAM-2, i.e., LFA-1 is so far known not affected by cytokines (27).

An increased expression, however, of an adhesion molecule is not required for an enhanced adhesion, because Kooyk et al. (28) demonstrated recently, that activation of T cells resulted in an enhanced adhesion, not by an increased expression of LFA-1, but by a modulation of the molecular conformation of LFA-1.

A number of other effects of IFN-γ can be enhanced by a synergism with TNF and/or IL-1 or vice versa on endothelial cells, including the enhancement of MHC class I and induction of MHC class II Ag (14). All these effect may contribute to the amplification of an immunologic reaction. The role of IFN-γ to implicate endothelium in an inflammatory process seems also relevant for the induction of the Shwartzman reaction. In the latter phenomenon, activation of endothelium and adhesion of neutrophils is known to play a role in pathophysiology (29).

Billiau et al. (30) clearly demonstrated a role of endogenous IFN-γ in the elicitation of the Shwartzman reaction using antibodies to IFN-γ (30).

In this study we demonstrated that the expression of the adhesion molecule ELAM-1 and the IL-6 production by endothelial cells can be modulated by the cytokine IFN-γ. The potentiating effect of IFN-γ described, in the induction of ELAM-1on human endothelial cells, may explain at least in part the difference between in vivo and in vitro dataon kinetics of ELAM-1 expression. However, other cytokines or a combination of cytokines, which undoubtedly circulate in vivo during an inflammatory reaction, may be also responsible for the prolonged expression of the adhesion molecule observed in vivo. Additionally, the enhancement by IFN-γ of the production of IL-6 may result in an amplification of the immune reaction.

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Chapter 4.3

LPS AND CYTOKINE-INDUCED ENDOTHELIAL CELL IL-6 RELEASE AND ELAM-1 EXPRESSION; INVOLVEMENT OF SERUM

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Summary

In this in vitro study, the influence of serum-concentration, heat inactivation of the serum and the origin of the serum on the responsiveness of cultured human umbilical vein endothelial cells (HUVEC) to immunological challenges was investigated. Addition of human serum during stimulation with 1μg/ml bacterial lipopolysaccharide (LPS) increased endothelial cell ELAM-1 expression and interleukin (IL)-6 release five to ten-fold. Full endothelial cell responsiveness to LPS required 10 to 50% human serum and was largely abrogated after heating the serum for 30 minutes to 56°C. Addition of newborn- or fetal bovine serum instead of human serum, induced even higher IL-6 release and ELAM-1 expression in response to LPS, whilst heat-inactivation of these serum-batches only moderately decreased endothelial cell responses. Endothelial cell IL-6 release and ELAM-1 expression after stimulation with IL-1β and tumor necrosis factor-α (TNF-α) were less influenced by heat inactivation of the serum and by omission of serum, whilst responses to PMA remained completely unaffected by such modifications in assay media. Finally, we demonstrated that also endothelial cell IL-8 release and ICAM-1 expression in response to LPS and cytokines were increased by addition of human serum, indicating that the use of serum-free assay media, or the use of media enriched by heat-inactivated (HI) human serum interferes with physiological endothelial cell responsiveness.

Although the origin of serum requirement for full endothelial cell responsiveness to LPS, IL-1β and TNF-α remains to be elucidated, these findings argue for the use of at least 10% not heat-inactivated serum as a medium-supplement during in vitro assays in which endothelial cells are used.

Introduction

The use of endothelial cell monolayers, grown on protein coated laboratory plastic has become a widely used model to study endothelial cell function in vitro. Little consensus exists on the choice of assay-media used in such studies.

Whilst some groups claim that 100% HI autologous human plasma, being the most physiological vehiculum, should be used (1), others choose buffered salt solutions enriched by glucose and bovine serum albumin as the standard medium. In most cases, assay media are positioned somewhere in between these two extremes, and contain 5 to 10% serum, which is, for economic reasons, often of bovine origin. Additional variation has risen from the habit of heat-inactivation of the serum. Heating serum for 30 minutes to 56°C inactivates complement factors C1, C2, C5, C8, C9 and factor B, thus preventing activation of the complement cascade.

Meanwhile, it has become clear that endothelial cell function depends on serum presence in culture medium. At less than 5% serum, endothelial cell proliferation is strongly reduced (2), endothelial cell monolayer integrity is perturbed (3, 4), and permeability of the monolayer increases (5).

Immunological responses of endothelial cells are altered in absence of serum. For example, TNF causes lysis of rat pulmonary endothelial cells cultured in serum free medium, which is prevented by presence of 5% bovine calf serum (BCS) (6).

Likewise, a more than tenfold increased prostaglandin E_2 and prostacyclin release by bovine pulmonary endothelial cells after TNF-α stimulation was measured when fetal bovine serum was added to serum-free medium (7). Occasionally, serum constituents such as hypoxanthine (8) have been identified as a factor responsible for altered endothelial cell function in presence of serum, but often it is even unclear whether one particular factor, or a combination of influences of serum is involved.

In this study, we investigated the influence of serum on endothelial cell responsiveness to LPS, and the LPS inducible cytokines TNF-α and IL-1β. LPS, TNF and IL-1 incite a number of pro-inflammatory events in endothelial cells (reviewed in 9). Increased and *de novo* expression of respectively ICAM-1 and ELAM-1, two endothelial cell adhesion molecules involved in leukocyte adherence, and the release of the inflammatory cytokines IL-6 and IL-8 were measured to define endothelial cell activation.

Materials and Methods

Reagents

Recombinant human (rh) TNF-α was kindly provided by BASF/Knoll Ag. (Ludwigshafen, FRG). rhIL-1β was a kind gift of Dr. S. Gillis (Immunex, Seattle, WA). LPS (E. Coli derived) and the phorbol ester PMA were purchased from Sigma (St. Louis, MO). Human serum was obtained from the local blood bank, pooled (unless mentioned otherwise), and either stored directly at -20°C, or heated to 56°C and maintained at that temperature for 30 min before storage (referred to as HI serum). Pooled human serum contained <5pg/ml endotoxin, as determined in the Limulus-assay (Kabi Coa-test, Kabi Diagnostica, Nykoping, Sweden).

Cells

HUVEC were obtained by collagenase treatment of the human umbilical vein and cultured in fibronectin-coated (fibronectin kindly provided by Dr. J. van Mourik, CLB, Amsterdam, The Netherlands) tissue culture flasks (Costar, Cambridge, MA) in culture medium. Culture medium was composed of RPMI-1640 (Gibco Europe, Paisley, Scotland), supplemented with 10% HI human serum, 10% HI bovine calf serum (BCS) (Hyclone, Logan, UT), 50μg/ml heparin (Sigma), 30μg/ml endothelial growth supplement (Collaborative Research Incorporated, Bedford, MA) and antibiotics.

Endothelial cells were characterized by their pavement-like monolayer morphology, by positive staining with mAb hec7 directed against PECAM-1 (10), generously provided by Dr. W.A. Muller, Rockefeller University, NY, and by positive staining with the anti ELAM-1 mAbs ENA1 and ENA2 (11) after 4 hour incubation with TNF-α.

Induction of ELAM-1 and ICAM-1 expression, and cytokine release.

HUVEC were seeded at 10^4 cells/well in fibronectin-coated 96-well flat-bottom tissue culture plates (Costar) in culture medium, one day prior to stimulation. HUVEC were rinsed twice with RPMI-1640 and the medium was replaced by the appropriate agents in RPMI-1640 supplemented with antibiotics, without growth factor, and with serum as indicated (and 0.1% BSA (endotoxin contamination <1ng/g, Sigma) in experiments in which serum-free medium was employed).

Supernatants were harvested and kept at -20°C until use in the IL-6 and IL-8 ELISA's.

Endothelial cell monolayers, were washed, fixed with 0.05% glutaraldehyde for 10 minutes at room temperature (RT), and kept at 4°C to determine cell-surface antigen expression.

IL-6 and IL-8 ELISA's

The culture supernatant IL-6 and IL-8 concentrations were determined using previously described sandwich-ELISA's for IL-6 (12) and IL-8 (13). In short, 96-well immuno maxisorp plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with murine mAb 8, specific for human IL-6 (14), kindly provided by Dr. L.A. Aarden (CLB, Amsterdam, The Netherlands), and polyclonal goat-anti IL-8 antiserum (5μg/ml) for the IL-6 and the IL-8 ELISA respectively. rhIL-6 (a generous gift from Prof. Dr. W. Sebald, Physiologisch-Chemisches Institut der Universität, Würzberg, FRG) and rhIL-8 were used for standard titration curves.

Test samples were added and incubated for 1.5 hours. Polyclonal rabbit anti-human IL-6 antiserum, followed by peroxidase conjugated goat anti-rabbit IgG (Jackson, Westgrove, PA), or biotin conjugated polyclonal goat anti-human IL-8 (2.5μg/ml), followed by peroxidase conjugated avidin (Bio-Rad, Richmond, CA) were used for the IL-6 and IL-8 ELISA respectively. O-Phenylenediamine (Sigma) was added as a substrate and photospectometry was performed at 492nm. Standard titration curves in the ELISA's were not influenced by presence of different amounts of untreated or HI serum in the sample buffer.

Detection of cell-surface antigens.

ELAM-1 and ICAM-1 expression were determined in ELISA's, using the ELAM-1 specific mAb ENA1 (11), and the ICAM-1 specific mAb RR1/1 (15), followed by per-

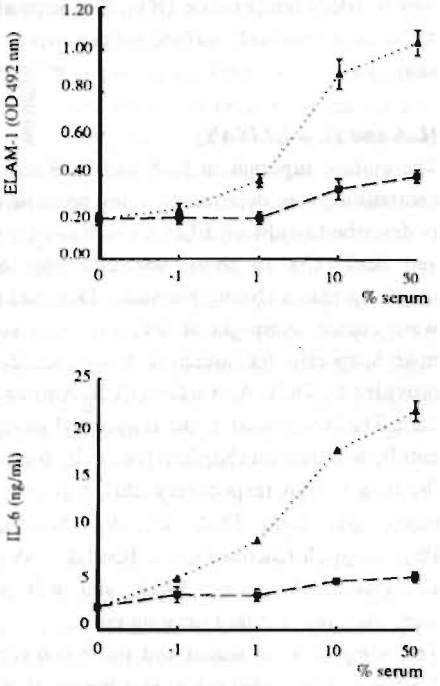

Figure 1: *Effects of different amounts of untreated (▲) and HI (■) human serum on ELAM-1 expression and IL-6 release induced by 1µg/ml LPS. HUVEC were incubated in presence of LPS for 18 hours in RPMI supplemented with 0.1% BSA and the indicated amount of serum. ELAM-1 expression and IL-6 release were measured as indicated in Materials and Methods. Spontaneous IL-6 release reached 0.2ng/ml in presence of both HI and untreated serum, whilst ELAM-1 expression remained below the detection limit of the assay. Data are expressed as mean and SD of four measurements.*

oxidase conjugated goat anti-rabbit IgG. O-Phenylene-diamine (Sigma) was added as a substrate and photospectometry was performed with a Microtiter ELISA reader. Results are expressed in optical density (OD) units at 492nm.

Results

Effect of serum on endothelial ELAM-1 expression and IL-6 release induced by LPS

Addition of human serum to basal medium (RPMI-1640 supplemented with 0.1% BSA and antibiotics), induced increases in both ELAM-1 expression and IL-6 release by HUVEC stimulated for 18 hours with 1µg/ml LPS in such medium *(Fig. 1)*.

IL-6 release was significantly increased by as little as 0.1% human serum ($P<0.01$, compared to 0% serum by the unpaired, two-tailed Student's *t*-test), whilst higher ELAM-1 expression was apparent at 1% or more human serum.

Microscopic evaluation revealed no increase in the number of pyknotic cells and no severe monolayer disruption during the first 24 hours of serumfree culture. In the same experiments the influence of human serum, heat-inactivated for 30 minutes at 56°C (HI serum), was assessed. Addition of 50% HI serum to basal medium increased LPS induced ELAM-1 expression as measured in ELISA from 0.19 to 0.40 (OD units at 492nm), whilst an OD 1.04 was reached in presence of untreated serum. IL-6 release was influenced even more by serum-presence (without serum 2.2ng/ml, in presence of 50% HI serum 5.3ng/ml, in presence of untreated serum 22ng/ml) *(Fig. 1)*.

To investigate whether heat-inactivation of the serum reduced the stimulating effect of serum on endothelial cell responsiveness, or whether an additional negative influence was induced by heat inactivation, 10% HI serum was added to the assay-medium and subsequently replaced step-wise by untreated serum. Clear positive effects on LPS induced

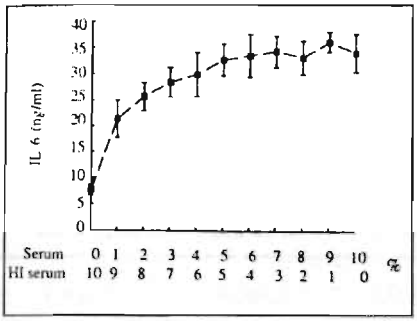

Figure 2: Effect of replacement of HI human serum by untreated serum on IL-6 release induced by 1µg/ml LPS. HUVEC were incubated in presence of LPS for 18 hours in RPMI supplemented with the indicated amounts of untreated and HI human serum. Spontaneous IL-6 release did not exceed 0.5ng/ml. Data are expressed as mean and SD of four measurements.

ELAM-1 expression (data not shown) and IL-6 release *(Fig. 2)* were induced by replacing 1/10 of the HI serum for untreated serum. In contrast, no significant effects were found after changing of 5/10 of untreated serum for HI serum, indicating that HI serum has no negative effect on endothelial cell responsiveness in this model.

Influence of HI and untreated human serum on ELAM-1 expression and IL-6 release, induced by LPS, TNF-α, IL-1β and PMA

To investigate whether the serum-effect interfered selectively with endothelial responses to LPS, we compared IL-1β, TNF-α and PMA induced ELAM-1 and IL-6 time-response curves with LPS induced responses. The kinetics of ELAM-1 expression were not influenced by serum-presence; a decrease in expression started after 6 hours, independent whether incubations were performed in presence of 10% HI human serum or untreated human serum *(Fig. 3)* or in absence of serum (data not shown).

Maximal differences of HI human serum and untreated human serum on ELAM-1 expression and IL-6 release were observed in response to LPS *(Fig. 3)*.

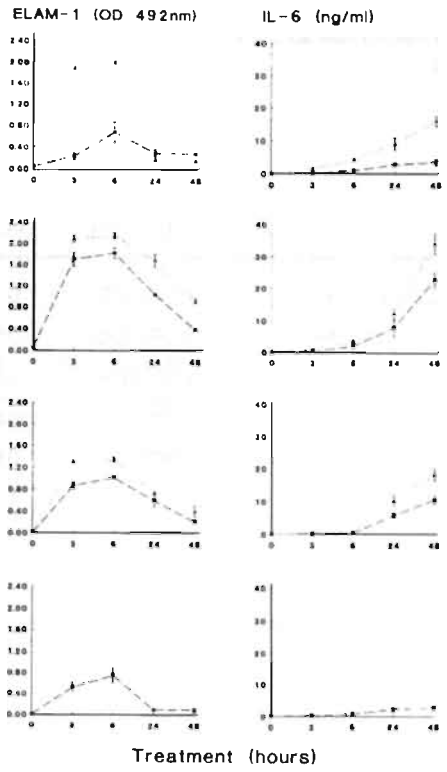

***Figure 3:** Kinetics of ELAM-1 expression and IL-6 release induced by 1µg/ml LPS, 1ng/ml TNF-α, 10u/ml IL-1β or 5ng/ml PMA in presence of untreated (▲) and HI (■) human serum. HUVEC were incubated for the time interval indicated in RPMI supplemented with 10% untreated or HI human serum in presence of the indicated agent. Spontaneous 48 hour IL-6 release reached 2ng/ml in presence of both HI and untreated serum, whilst 6 hour ELAM-1 expression remained below the detection limit. Data are expressed as mean and SD of four measurements.*

Table 1

Comparison of the effects of HI and untreated human serum on ICAM-1 expression and IL-8 release induced by LPS, IL-1β and TNF-α.

	ICAM-1 (OD 492nm)		IL-8 (ng/ml)	
	HI serum	untr. serum	HI serum	untr. serum
unstimulated	0.33±0.02	0.43±0.02	1.3±0.3	4.7±0.3
LPS 10ng/ml	0.39±0.02	0.59±0.02	3.6±0.4	14 ±3
LPS 1ug/ml	0.77±0.04	0.87±0.01	39 ±3	73 ±7
IL-1β 10U/ml	0.75±0.02	0.81±0.01	65 ±6	79 ±7
IL-1β 500U/ml	0.97±0.03	0.94±0.01	116 ±3	134 ±5
TNF-α 1ng/ml	1.05±0.03	1.10±0.02	130 ±10	150 ±6

HUVEC were incubated for 18 hours in RPMI supplemented with 10% untreated or HI human serum in presence of the indicated agent. Data are expressed as mean ± SD of four measurements

Endothelial cell responsiveness to TNF and IL-1 was, however, also increased by serum-presence. Addition of 10% HI human serum to basal medium (data not shown), as well as replacing HI serum for untreated serum *(Fig. 3)* consistently increased both IL-1β and TNF-α induced endothelial cell ELAM-1 expression and IL-6 release, although such increases were generally below 100%. PMA induced ELAM-1 expression and IL-6 release remained unaffected by either addition of HI serum or untreated serum to basal medium.

Heat-inactivated (100°C, 30 minutes) preparations of IL-1, TNF and PMA did not induce IL-6 release or ELAM-1 expression, indicating that the described influences of serum on TNF and IL-1 provoked responses were not caused by endotoxin contamination (data not shown).

Influence of HI and untreated human serum on endothelial IL-8 release and ICAM-1 expression

To investigate whether serum-presence interfered selectively with endothelial IL-6 release and ELAM-1 expression, we investigated two other parameters of endothelial cell activation, IL-8 release and ICAM-1 expression. Both IL-8 release and ICAM-1 expression, induced by stimulation with low amounts of LPS, were increased by addition of 10% HI human serum to basal medium (data not shown) as well as by replacing HI serum for untreated serum *(Table 1)*.

Lower but consistent differences between untreated and HI serum were also found

Table 2.

Comparison of human sera from different individuals and commercial bovine sera preparations on their influence on LPS induced HUVEC IL-6 release and ELAM-1 expression

	IL-6 (ng/ml)				ELAM-1 (OD 492nm)			
	HI serum		untr. serum		HI serum		untr. serum	
	exp. 1	exp. 2	exp. 1	exp. 2	exp. 1	exp. 2	exp. 1	exp. 2
HS-1	0.3±0.1	2.0±0.1	4.6±0.2	18 ±4	0.08±0.03	0.16±0.01	0.48±0.06	0.51±0.02
HS-2	0.3±0.1	2.6±0.7	4.0±0.2	22 ±4	0.19±0.07	0.13±0.01	0.57±0.07	0.50±0.02
HS-3	1.2±0.3	8.3±0.4	5.4±0.5	47 ±3	0.27±0.06	0.30±0.01	0.66±0.05	0.48±0.02
HS-4	1.0±0.3	6.1±0.1	4.6±0.4	38 ±4	0.11±0.02	0.14±0.02	0.28±0.03	0.26±0.01
HS-5	0.4±0.1	4.2±0.4	4.1±0.2	18 ±3	0.19±0.03	0.21±0.02	0.65±0.02	0.57±0.11
HS-6	0.4±0.1	4.6±0.3	6.3±0.9	34 ±2.6	0.27±0.12	0.27±0.03	0.81±0.06	0.58±0.10
BCS Hyclone-1	1.6±0.1	8.1±0.5	6.1±0.4	19 ±2	0.67±0.04	0.50±0.01	1.2 ±0.04	0.71±0.01
BCS Hyclone-2	1.7±0.1	15 ±1	6.5±0.9	39 ±3	0.74±0.04	0.54±0.02	1.2 ±0.10	0.76±0.03
NCS Gibco	5 ±0.9	65 ±7	10 ±2.0	57 ±3	1.2 ±0.11	0.72±0.02	1.2 ±0.13	0.62±0.04
FCS Hyclone-1	7.5±1.6	56 ±4	10 ±1.6	50 ±4	1.3 ±0.08	0.81±0.02	1.4 ±0.10	0.69±0.02
FCS Gibco	6.8±1.1	56 ±8	10 ±2.1	52 ±6	1.5 ±0.07	0.87±0.01	1.5 ±0.07	0.79±0.03
FCS Hyclone-2	6.8±0.8	57 ±8	8.2±1.9	43 ±4	1.4 ±0.10	0.79±0.03	1.3 ±0.07	0.67±0.03

HUVEC were stimulated with 1μg/ml LPS for 18 hours in RPMI supplemented with 10% untreated or HI serum of the indicated origin. Data from two separate experiments are shown, indicating mean ± SD of quadruplicates.

using 1μg/ml LPS, and IL-1β and TNF-α as stimuli, except for ICAM-1 expression after strong stimulation (500U/ml IL-1β and 1ng/ml TNF). Unstimulated HUVEC expresses low levels of ICAM-1 (16) and releases small amounts of IL-8. These basal levels of ICAM-1 expression and IL-8 release were also significantly increased by exchanging HI serum for untreated serum ($p<0.01$ as compared by the unpaired, two-tailed Student's *t*-test).

Comparison of sera from different individuals, and commercial preparations of BCS, NCS (newborn calf serum) and FCS (fetal calf serum).

Six human sera, obtained from healthy adult donors of the local blood bank, and standard commercial bovine sera preparations of BCS, NCS and FCS, obtained from Gibco and Hyclone, were compared on their influence on endothelial cell responsiveness. HUVEC IL-6 release and ELAM-1 expression after 18 hours of stimulation with 1μg/ml LPS in presence of 10% HI and untreated serum were measured. Consistent lower HUVEC responses were measured after HI of the human sera, although there was some inter-individual and inter-assay variation in absolute levels of IL-6 release and ELAM-1 expression *(Table 2)*.

Sera of bovine origin generally induced higher IL-6 and ELAM-1 responsiveness than human serum. In contrast to human serum, heat inactivation of BCS only slightly reduced endothelial cell responsiveness, whilst consistent heat inactivation induced reductions were not observed with NCS or FCS. None of the sera-batches used, induced significant spontaneous IL-6 release or ELAM-1 expression (data not shown).

Discussion

We demonstrate in this study that HUVEC ELAM-1 expression and IL-6 release, induced by stimulation with LPS, were increased by addition of 0.1% to 1% human serum, and showed five to ten times higher levels after addition of 50% human serum to BSA containing basal medium. Addition of human serum, heat-inactivated for 30 minutes at 56°C, had less response enhancing effects. Since presence of HI human serum instead of untreated serum did not seriously interfere with other endothelial cell functions, such as responses on PMA, monolayer integrity (by microscopic evaluation) and endothelial cell proliferation (2), and since no evidence was found for a negative influence of HI serum on endothelial cell responsiveness, we conclude that one or more heat labile serum factors in human serum specifically enhance endothelial cell responses to LPS, and the cytokines TNF-α and IL-β. This effect of human serum appeared to involve a direct effect on the endothelial cell, since also in absence of a stimulus, ICAM-1 expression and IL-8 release were up-regulated by presence of 10% untreated human serum. Furthermore, a serum induced increase in the biological activity of TNF-α and IL-1β is unlikely, since endothelial ELAM-1 expression and IL-6 release induced by addition of anti TNF-receptor antibody htr-9, known to mimic TNF-activation by cross-linking TNF-receptors on endothelial cells (17), also were higher in presence of untreated human serum than in presence of HI serum (data not shown).

Protein kinase C activation by phorbol esters induces IL-6 release (18) and ELAM-1

expression (11) by endothelial cells. Protein kinase C activation is, however, not involved in TNF induced ELAM-1 expression on endothelium (19). Cytokine induced IL-6 release by endothelial cells might also be induced independently from protein kinase C, since IL-6 gene activation in fibroblast can be induced independently from protein kinase C (20). Since we found no influences of human serum on endothelial cell responses to the protein kinase C activating phorbol ester PMA, we conclude that serum-presence selectively enhances protein kinase C independent endothelial cell activation.

Untreated human serum increased LPS induced endothelial cell responses stronger than responses to IL-1β and TNF-α. The additional enhancing effect of serum presence on LPS-stimulation might depend on specific interactions between bacterial LPS and serum, changing the biological activity of LPS. Serum causes an alteration of the association state of LPS, by binding of LPS to the lipid-fraction in serum, and by an interaction with specific LPS binding proteins (21). Several LPS binding proteins, some of which unstable at 56°C, have been described in different animals (22-25).

Variation in the presence and the nature of such proteins might have contributed to the difference in influence of human serum and bovine sera batches, and the lack of reduction in serum effects after heat inactivation of bovine sera described in this study. Wright and co-workers recently showed that a 55kD human acute phase protein binds to LPS (26), and that the interaction of this complex with membrane CD14 is a crucial step in the activation of macrophages (27) and neutrophil granulocytes (28) by LPS.

Whether this LPS binding protein and CD14 are involved in endothelial cell responses on LPS in presence of serum is currently being investigated. Identification of a serum protein involved in enhancing endothelial cell responses to LPS could offer new insights in the regulation of host responses to microbial challenge, and in the pathophysiology of related syndromes such as septic shock, multiple organ failure and the acute respiratory distress syndrome.

We demonstrated in this study that omittance of serum from the medium, heat inactivation of human serum, and the use of bovine sera instead of human serum influenced endothelial cell responsiveness. These findings argument for the use of at least 10% untreated human serum as a medium-supplement during in vitro experiments in which human endothelial cells are used, to avoid interference with physiological endothelial cell responsiveness.

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Chapter 4.4

ANTI-CD14 ANTIBODIES REDUCE RESPONSES OF CULTURED HUMAN ENDOTHELIAL CELLS TO ENDOTOXIN

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Summary

LPS activates both myeloid cells and endothelial cells. Whereas CD14 has shown to be involved in LPS recognition by myeloid cells, the mechanism responsible for the strong response of endothelial cells to LPS remains to be elucidated. The role of CD14 in this process was studied using CD14 specific Ab. Anti-CD14 Ab inhibited LPS-induced IL-6 release and E-selectin expression by cultured human umbilical vein endothelial cells (HUVEC). mRNA encoding IL-6 and E-selectin was reduced in parallel. The inhibitory effect of anti-CD14 Ab was epitope dependent, maximal at low LPS concentrations and dropped with increasing LPS dose. Anti-CD14 Ab did not affect endothelial cell activation induced by IL-1ß, TNF-α and PMA. HUVEC IL-6 release and E-selectin expression were strongly reduced when LPS-activation was performed in absence of serum, indicating involvement of serum-components in LPS-activation of HUVEC. Nevertheless, anti-CD14 Ab blocked LPS-induced HUVEC activation also in absence of serum. Although the role of serum-components in LPS-activation remains to be elucidated, CD14 seems to be a key-mediator in LPS-induced activation of endothelial cells.

table 1

Anti-CD14 antibodies can inhibit LPS-induced responses of both HUVEC and human monocytes

	HUVEC		human monocytes	
	IL-6 (ng/ml)	E-selectin (A 492nm)	TNF (ng/ml)	IL-6 (ng/ml)
unstimulated	0.1 ± 0.1	0.02 ± 0.02	0.02 ± 0.02	0.3 ± 0.1
LPS	11.2 ± 0.7	1.6 ± 0.08	1.4 ± 0.2	8.4 ± 0.5
LPS + MEM-18	0.9 ± 0.2	0.12 ± 0.04	0.1 ± 0.1	0.04 ± 0.01
LPS + Cris6	0.5 ± 0.2	0.09 ± 0.04	0.1 ± 0.1	0.02 ± 0.01
LPS + UCHM1	2.7 ± 0.7	0.28 ± 0.05	0.87 ± 0.08	5.0 ± 0.4
LPS + MEM-15	1.2 ± 0.2	0.22 ± 0.04	0.50 ± 0.04	3.2 ± 0.4
LPS + BL-467	12.4 ± 1.5	1.6 ± 0.1	0.97 ± 0.05	7.2 ± 0.6
LPS + rabbit anti-CD14	1.4 ± 0.3	0.19 ± 0.05	0.12 ± 0.07	1.5 ± 0.1
LPS + MOPC21	10.8 ± 1.0	1.5 ± 0.1	1.2 ± 0.2	8.3 ± 0.4

Influence of 10 μg/ml mAb and rabbit polyclonal antibodies reactive with CD14 on HUVEC IL-6 release and E-selectin expression and monocyte TNF and IL-6 release induced by LPS. MOPC21, a non-reactive mAb of the IgG_1 subclass,was used as a control. Cells were incubated in presence of LPS (monocytes with 100 pg/ml and HUVEC with 1 μg/ml LPS) and antibodies for 5 hours (to determine maximal E-selectin expression) and 18 hours (to determine cytokine release). Data are expressed as mean ± SD of four measurements.

primer labeling kit of Boehringer Mannheim (Boehringer Mannheim, Mannheim, FRG) and hybridized to the blot (10^6 cpm/ml) as described (28). The IL-6 probe (1.3 kb HIII-EcoRI fragment) was kindly provided by Dr. Aarden (CLB, Amsterdam, Netherlands), the E-selectin probe (1.0 kb EcoRI-BglII fragment) was kindly provided by Celltech andthe actin probe (1.3 Pst fragment) was kindly provided by Dr. Berkvens (University of Leiden, Leiden, The Netherlands). Labeled bands were visualized by autoradiography.

Results

Anti-CD14 antibodies inhibit LPS-induced HUVEC responses

HUVEC-monolayers were incubated with 1 μg/ml LPS in RPMI-1640 enriched with 10% HS and antibiotics. The influence of coincubation with five murine anti-CD14 mAb, MEM-18, Cris6, UCHM1, MEM-15 and BL-467, and of serum of CD14 immunized rabbits (IgG concentration 10 μg/ml), on IL-6 release and on E-selectin expression by HUVEC was investigated.

MEM-18, Cris6, MEM-15 and rabbit polyclonal anti-CD14 antibodies prevented LPS

induced IL-6 release and E-selectin expression for 80% or more. UCHM1 inhibited HUVEC responses less effectively, whilst BL-467 as well as a nonreactive control mAb did not influence HUVEC responses. *(Table 1)*.

As a control, the same panel of antibodies was tested for their influence on 100 pg/ml LPS induced TNF and IL-6 release by human monocytes. The LPS-induced release of both cytokines was completely prevented by coincubation with MEM-18 and Cris6, whilst MEM-15 and rabbit anti-CD14 antibodies reduced both TNF and IL-6 release for more than 60% *(Table 1)*.

In parallel to the effect on LPS-induced HUVEC activation, mAb BL-467 hardly influenced LPS-induced monocyte TNF and IL-6 release *(Table 1)*. None of the anti-CD14 antibodies induced HUVEC IL-6

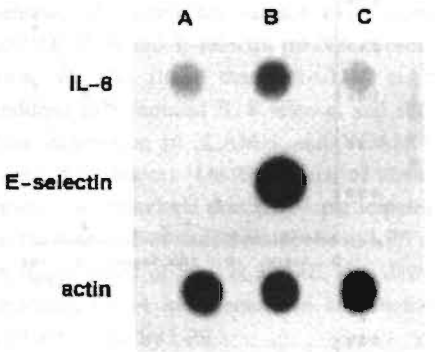

Figure 1: *MEM-18 inhibits LPS-induced IL-6 and E-selectin mRNA expression in HUVEC. mRNA was isolated from untreated HUVEC (A) and HUVEC stimulated for 2 hours with 1 µg/ml LPS in absence (B) presence (C) of 5 µg/ml MEM-18, in medium containing 10% HS. Parallel blots were run and hybridized with actin probe to compare the amount of RNA of different samples.*

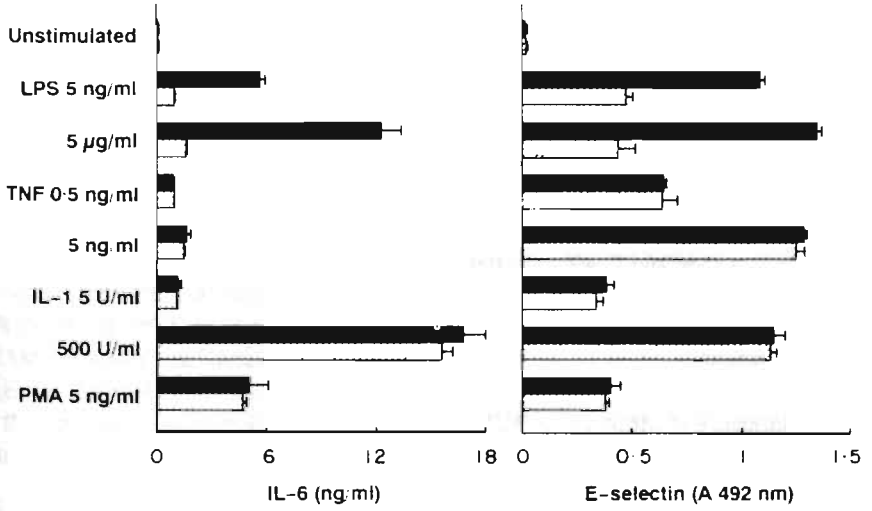

Figure 2: *MEM-18 inhibits LPS-induced, but not TNF, IL-1β and PMA-induced IL-6 release and E-selectin expression. HUVEC were incubated with the indicated reagents in absence (■) and presence (////) of 5 µg/ml MEM-18. E-selectin expression and IL-6 release were measured after respectively 5 and 18 hours as indicated in Material and Methods. Data are expressed as mean and SD of four measurements.*

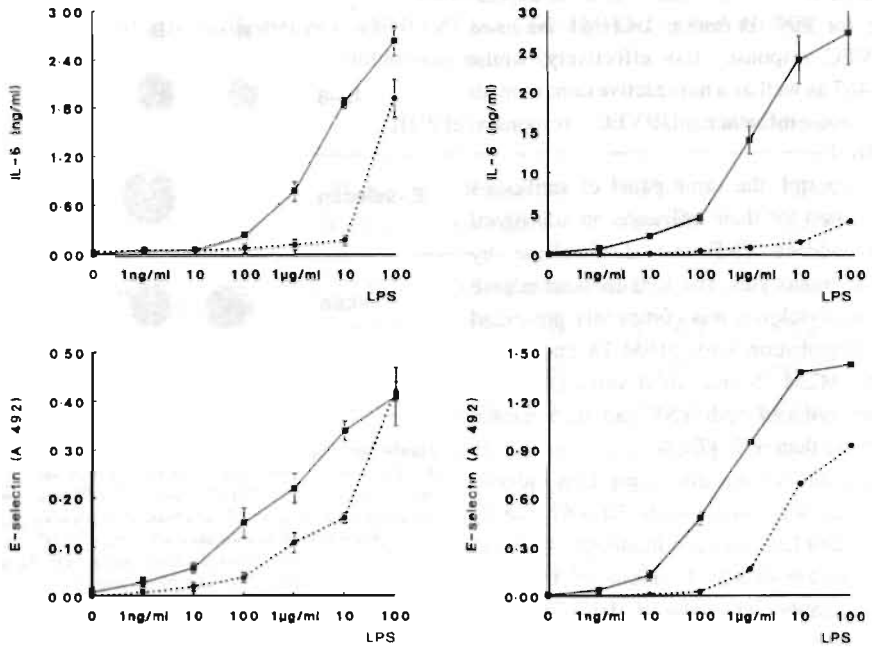

Figure 3: *MEM-18 inhibits LPS-induced IL-6 release and E-selectin expression both in absence (left graphs) and presence (right graphs) of 10% HS. HUVEC were incubated with different concentrations of LPS with (●) and without (■) of 5 µg/mlMEM-18 for 5 hours (to determine maximal E-selectin expression) and 18 hours (to determine IL-6 release) in presence or absence of serum. Data are expressed as mean and SD of four measurements. If SD bars are not shown, they fall within the symbol.*

release, HUVEC E-selectin expression and monocyte cytokine release in absence of LPS (data not shown). MEM-18, which inhibited HUVEC IL-6 release and E-selectin expression at 0.2 µg/ml, with a maximal inhibition at 2 µg/ml (data not shown) was selected for further experiments.

Next, the influence of MEM-18 on HUVEC IL-6 and E-Selectin mRNA expression induced by incubation with 1 µg/ml LPS for 2 hours, was determined. In presence of MEM-18, substantially reduced amounts of mRNA encoding IL-6 and E-selectinwere detected *(Figure 1)*, indicating interference of MEM-18 with LPS induced IL-6 release and E-selectin expression on a pre-transcriptional level. To investigate whether anti-CD14 antibodies specifically inhibited HUVEC responses to LPS, we compared HUVEC responses to LPS, IL-1ß, TNF-α and PMA in presence and absence of MEM-18. Whereas MEM-18 induced a major reduction E-selectin expression and IL-6 release in response to 5 ng/ml LPS and 5 µg/ml LPS, no clear influence on responses of HUVEC monolayers stimulated with IL-1ß, TNF-α and PMA was found *(Figure 2)*.

Inhibition of LPS induced HUVEC IL-6 release and E-selectin expression is notabrogated in absence of serum

To investigate whether serum-presence is a prerequisite for CD14 mediated LPS-activation of HUVEC, HUVEC monolayers were rinsed intensively with serum-free medium before addition of LPS, to assure maximal removal of soluble serum-components. Monolayers were subsequently incubated in presence or absence of 10% HS with LPS concentrations ranging from 1 ng/ml to 100 µg/ml in RPMI-1640 supplemented with 0.1% BSA. In presence of serum, MEM-18 reduced IL-6 release and E-selectin expression in response to 1-100 ng/ml LPS for 90% or more. This reduction was partly overcome by stimulation with higher concentrations of LPS *(Figure 3)*. As reported previously (29) HUVEC showed diminished responses to LPS in absence of serum *(Figure 3)*. MEM-18, however, still effectively reduced both LPS induced IL-6 release and E-selectin expression in absence of serum in the incubation-medium *(Figure 3)*. The use of special serum free endothelial cell culture medium (Endothelial-SFM, Gibco) instead of RPMI supplemented with BSA, and additional attempts to remove serum proteins, either by repeating the wash-procedure or by 24 hour culture in serum-free medium before washing and activation in new serum free medium, did not prevent the inhibitory effects of MEM-18 coincubation in the above described experiment (data not shown).

Discussion

We demonstrated that certain anti-CD14 Ab inhibited LPS-induced activation of cultured HUVEC, measured by induction of (1) IL-6 release; (2) E-selectin surface expression, and (3) IL-6 and E-selectin mRNA expression. We also found that anti-CD14 mAb reduced LPS-induced IL-8 release, and surface expression of ICAM-1 and VCAM-1 (data not shown). On the basis of these results, we conclude that CD14 participates in the response of endothelial cells to LPS.

A recent study of Frey et al (30), also demonstrates CD14 involvement in endothelial cell activation by LPS.

This finding extends the immunological importance of CD14 in terms of its role in LPS-induced activation of not only hemopoietic, but also non-hemopoietic cells. We and others found clear differences in the capacities of anti-CD14 Ab to block LPS induced cellular activation (5, 30). Both differences in epitope-specificity and differences in affinity of the individual anti-CD14 Ab could have contributed to this variation.

Capacities of anti-CD14 Ab to block LPS-induced HUVEC activation were, however, similar to the capacities of these Ab to block LPS-induced activation of monocytes, indicating that the same CD14 epitope is involved in the activation process. The finding that anti-CD14 mAb do not inhibit endothelial cell responses to cytokines and PMA demonstrates that CD14 is specifically involved in the LPS-induced activation.

There are still several questions to be answered. First, what is the role of CD14 in cellular activation by LPS? The fact that anti-CD14 mAb are able to inhibit LPS-induced activation of HUVEC in the absence of serum supports the concept that CD14 is able to interact directly with LPS (7, 8, 11, 12, 30). It can, however, not be excluded that

some serum proteins like LBP may remain bound to the endothelial cell surface and mediate LPS-CD14 interactions, despite the efforts to wash theseproteins away. Another conclusion drawn from this observation is that CD14 must be associated with the endothelial cell membrane.

A next question regards the origin of CD14 present on endothelial cells. One possibility is that CD14 represents an endogenous membrane molecule. Since soluble CD14 is found in high amounts in normal plasma (22, 31), association of exogenous CD14 with a counter-receptor on the surface of endothelial cells would be another option, which has recently been claimed (30). Attempts to prevent association of exogenous CD14 with the endothelial cell surface by culturing HUVEC monolayers under serum-free conditions before LPS-activation did, however, not prevent the inhibitory effect of anti-CD14 mAb. Whatever the answer is, the quantity of cell surface-associated CD14 on HUVEC seems to be fairly low, since we were not able to detect surface CD14 using either flow cytometry analysis or precipitation from detergent lysate of surface iodinated HUVEC (data not shown).

In contrast, we obtained positive results using both these methods for detection of surface CD14 on monocytes (data not shown). However, in addition to the functional evidence for the presence of CD14 on endothelial cells, weak staining of endothelial cells by anti-CD14 mAb has been reported (19-21). Finally, it is not clear which serum proteins are involved in CD14-dependent activation of endothelial cells in the presence of serum. For monocytes, LBP and 'septin' have been identified as serum-components which enhance CD14 mediated LPS activation (5, 6, 10). The fact that the LPS-induced response of endothelial cells in presence of serum is much stronger compared to in serum-free conditions, clearly indicates that serum factors such as LBP participate in this process. The experiments addressing the questions mentioned above are currently being addressed in our laboratory.

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Chapter 5.1

TUMOUR NECROSIS FACTOR-α INDUCES NEUTROPHIL MEDIATED INJURY OF CULTURED HUMAN ENDOTHELIAL CELLS

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Scand. J. Immunol. 1991, 34: 197-206

Summary

We investigated the ability of TNF-α to mediate damage of endothelial cells in presence of neutrophils, by measuring detachment of cultured human umbilical vein endothelial cells (HUVEC). Endothelial cell detachment was increased from 5% to about 75% by presence of 1 to 10 ng/ml TNF-α during incubation with neutrophils, whereas negligible endothelial cell lysis was observed as measured by ^{51}Cr release. TNF-α was compared with the cytokines IL-1α and IFN-γ and with PMA and LPS. Both TNF-α and PMA appeared to be strong triggers for neutrophil induced endothelial cell detachment, whilst reduced injury was seen after addition of IL-1α and LPS. IFN-γ did not induce endothelial cell detachment, but potentiated the effect of both TNF-α and IL-1α. TNF-α induced endothelial cell detachment was neutrophil dependent, since preincubation of neutrophils, but not preincubation of endothelial cells with TNF-α, caused endothelial cell detachment. Thus, TNF-α induced increase in neutrophil-adhesiveness of HUVEC was found not to be essential for endothelial damage. Preincubation of neutrophils in suspension with TNF-α, induced rapid activation, followed by nearly complete deactivation of neutrophils, as measured by their capacity to induce detachment of endothelial cells after removal of TNF-α.

These results indicate, that local presence of TNF-α might be critical in tissue or organ damage during early, neutrophil mediated inflammatory processes, independent of enhanced adhesiveness of endothelium for neutrophils.

Introduction

Neutrophil infiltration is a general pathophysiological finding in early inflammatory tissue or organ damage. Local release of TNF-α, together with other cytokines such as IL-1 and IFN-γ, can be incited by microbial products, as well as by nonspecific stress, resulting from short term ischaemia during organ transplantation procedures, vascular surgery, or severe circulatory shock (1, 2). These cytokines can induce several pro-inflammatory events in the interaction between neutrophils and endothelium (reviewed in 3).

Endothelial cells respond to TNF-α and IL-1 with increased and de novo expression of the adhesion molecules ICAM-1 and ELAM-1 (4-6) leading to increased neutrophil adherence and rapid neutrophil transmigration (7, 8). Simultaneously neutrophils can be activated directly by TNF-α and IL-1 (9-13), and indirectly via the release of IL-6 (14, 15), IL-8 (16), GM-CSF (11, 17, 18) and platelet activating factor (PAF) (19, 20) by endothelial cells upon TNF-α and IL-1 stimulation.

Whether neutrophil-endothelial interaction in presence of TNF-α or other cytokines induces direct damage to endothelial cells, still has to be revealed.

Prolonged exposure to TNF-α or IL-1α is not directly cytotoxic for endothelial cells cultured in vitro (21, 22). However, local endothelial cell damage can be incited in experimental animals by local TNF-α plus IL-1α administration during the Shwartzman reaction (23) and by systemic TNF-α addition after previous methylcholanthrene induced tumor growth (24). Prolonged continuous subcutaneous perfusion of high amounts of TNF-α in mice, can induce local necrosis (25). Tissue and endothelial cell damage, observed in these experiments, is accompanied by local accumulation of neutrophils. These cells are considered to play an important role in endothelial cell damage.

The aim of this study was to investigate the role of TNF-α in neutrophil induced endothelial cell damage in vitro. Capacities of TNF-α, compared to IL-1α, IFN-γ, LPS, and PMA, in inducing neutrophil mediated injury of HUVEC, were determined. Furthermore, we demonstrated that preincubating of neutrophils, but not preincubation of endothelial cells with TNF-α, increases endothelial cell damage.

Materials and Methods

Reagents

Recombinant (r) human (h) TNF-α was kindly provided by BASF/Knoll Ag. (Ludwigshafen FRG). rhIL-1α was a kind gift of Dr. S. Gillis, Immunex, Seattle, WA. rhIFN-γ (E. Coli derived) was a gift from Dr. P. Van der Meide, TNO, Rijswijk, The Netherlands. LPS (E. Coli derived) and PMA were purchased from Sigma (St. Louis, MO).

Cells

HUVEC were obtained by collagenase treatment of the human umbilical vein. The cells were cultured in fibronectin-coated (fibronectin kindly provided by Dr. J. van Mourik, CLB, Amsterdam, The Netherlands) tissue culture flasks (Costar, Cambridge, MA) in RPMI1640 (Gibco Europe, Paisley, Scotland), supplemented with 10% heat-inactivated human serum, 10% heat-inactivated bovine calf serum (BCS) (Hyclone, Logan, UT), 50μg/ml heparin (Sigma), 30μg/ml endothelial growth supplement (Collabora-

tive Research Incorporated, Bedford, MA) and antibiotics. Endothelial cells were characterized by their pavement-like monolayer morphology, by positive staining with Mab hec7 directed against PECAM-1 (26), generously provided by Dr. W.A. Muller, Rockefeller University, NY, and by positive staining with the anti ELAM-1 Mab ENA2 (27) after 4h incubation with TNF-α.

Neutrophil leukocytes were prepared from buffy coats of donor blood kindly provided by the local blood bank, using standard techniques of buoyant density centrifugation on Lymphoprep (Nycomed, Oslo, Norway). Neutrophils were separated from erythrocytes by density gradient centrifugation on percoll (1.08g/ml, Pharmacia, Uppsala, Sweden). Preparations of neutrophils were >95% pure, as evaluated after May Grunwald Giemsa staining.

Preincubation of neutrophils with TNF-α was performed by incubating neutrophils in polypropylene tubes at 10^7cells/ml in RPMI1640 10%BCS with 10ng/ml TNF-α for the time-interval indicated. Subsequently neutrophils were rinsed twice with ice-cold RPMI1640 1%BCS, and added to the monolayers. Control cells were kept at 4°C during this period.

To determine cell-loss by the preincubation-procedure, tubes were incubated with EDTA-containing lavage-medium for an additional 10 minutes at 37°C, to detach remaining adherent neutrophils. Neutrophil-loss was determined separately for each tube, by measuring total cell-numbers in pooled waste-media and lavage-medium, and never exceeded 10%.

Injury assays

^{51}Cr-release and detachment assays were performed essentially as described by Harlan et al. (28). HUVEC (passage 3) were grown to confluence during 24h in fibronectin-coated 96-well, flat bottom, microtiter plates (Costar). Final plating density was ± $2*10^4$ cells/well (well-surface: 0.32cm^2). The last 16h of this culture Chromium-51 as sodium [^{51}Cr]chromate was added to the wells (18kBq/well, specific activity 11GBq/μg Cr). At the beginning of the assay monolayers were carefully washed by five successive exchanges of 200μl/well RPMI1640 1%BCS.

Neutrophils and reagents in RPMI1640 supplemented with 10%BCS were added to the monolayer as indicated, at a final volume of 200μl/well, and incubations were performed at 37°C for 3h, unless mentioned otherwise. Endothelial cell lysis was determined by measuring ^{51}Cr release in 100μl cell-free supernatant medium, removed from each well with care not to disturb the monolayer. Next, 100μl of fresh medium was added to the remaining 100μl in each well. Detached endothelial cells were suspended by repeated careful pipetting, and 100μl of this suspension was removed to determine detachment (these samples thus contained 50% of the amount of ^{51}Cr in the lysis samples, plus the ^{51}Cr present in the detached cells).

Control monolayers remained visually confluent after performing this procedure. Maximal ^{51}Cr content was determined in wells receiving 100μl of 1N NaOH, and reached about 10^5dpm. All samples were counted in a gamma-spectrophotometer.

Detachment values were calculated by subtracting half of the ^{51}Cr counts measured in the lysis samples drawn from the same wells,

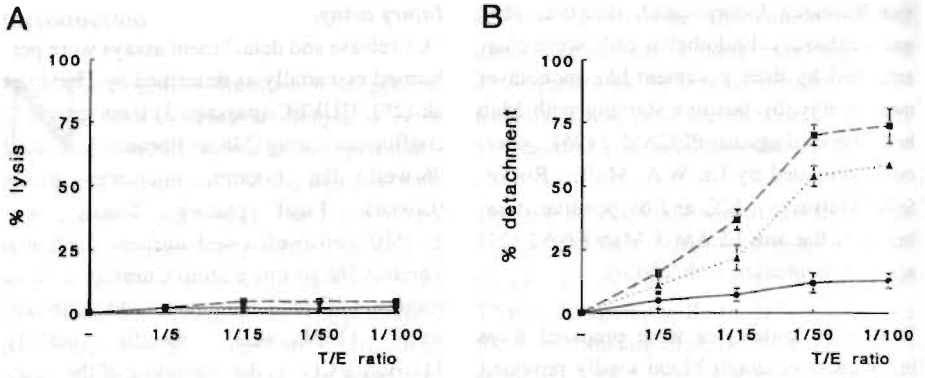

Fig. 1. Neutrophil mediated HUVEC lysis (A) and HUVEC detachment (B), induced by 10ng/ml TNF-α (■), 50ng/ml PMA (▲), or without stimulation (●), at different T/E ratios. HUVEC injury was measured after 3h of incubation in presence of effector-cells and agents. A representative experiment out of a series of 4 is shown. Data indicate mean ± SD of four measurements.

from the ^{51}Cr counts measured in the detachment samples. Furthermore, measurements of endothelial cell lysis and endothelial cell detachment were corrected for non-specific ^{51}Cr content, by subtracting ^{51}Cr counts measured in lysis/detachment samples from wells without effector cells (in these wells spontaneous ^{51}Cr release and endothelial cell detachment ranged from 5 to 10% of total ^{51}Cr content).

Results

Effect of TNF-α and PMA on neutrophil induced endothelial cell injury

Neutrophils were analyzed for their capacity to induce lytic injury or non-lytic detachment of HUVEC. Neither presence of TNF-α nor presence of PMA during a 3h assay at 37°C in medium containing 10% BCS, induced substantial endothelial cell lysis in presence of neutrophils, as measured by ^{51}Cr release from HUVEC *(Fig. 1A)*. In our hands, substantial short term endothelial cell lysis only occurred by using IL-2 cultured lymphocytes as effector cells, or by performing the assay in absence of serum (and presence of 0.5% BSA) (data not shown).

To determine the capacity of neutrophils to affect endothelial cell monolayer integrity, we measured detachment of endothelial cells from HUVEC monolayers, after applying mild shear stress by repeated pipette suction, as a parameter for early cell damage (18). Unstimulated neutrophils induced a minor increase in endothelial cell detachment. Both TNF-α and PMA induced a clear target effector (T/E) ratio dependent increase in endothelial cell detachment, which leveled off at a T/E ratio of 1/50, reaching 74% and 58% detachment in presence of respectively 10 ng/ml TNF-α and 50ng/ml PMA *(Fig. 1B)*. Next, detachment was measured after different time-intervals, at a T/E ratio of 1/50. TNF-α caused a rapid increase in endothelial cell detachment, which did not increase further after 2h *(Fig. 2)*. PMA, was maximal effective in triggering detachment at a con-

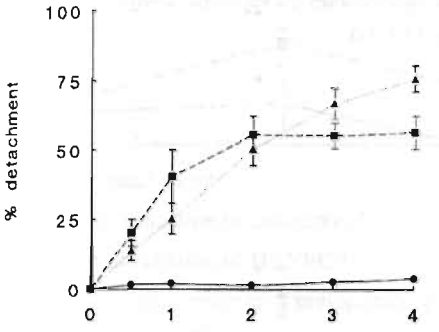

Fig. 2. Time course of TNF-α (■) and PMA (▲) induced neutrophil mediated HUVEC-damage, compared to detachment induced by unstimulated neutrophils (●). Neutrophils were added to HUVEC-monolayers (T/E = 1/50), and incubated together with the stimulus for the time-interval mentioned.
A representative experiment out of a series of 3 is shown. Data indicate mean ± SD of 4 measurements.

centration between 5 and 50 ng/ml (data not shown), and induced a continuous increase in endothelial cell detachment over the first 4h *(Fig. 2)*.

Comparison of the cytokines TNF-α, IL-1α and IFN-γ, and of LPS on neutrophil mediated endothelial cell damage

Several concentrations of known neutrophil activating agents were tested for their influence on neutrophil mediated endothelial cell detachment after 3h of coincubation *(Fig. 3)*. TNF-α induced maximal endothelial cell detachment at a concentration of 10 ng/ml, but showed already a clear effect at 100 pg/ml. IL-1α, in a range of 10-1000 u/ml, induced endothelial cell detachment, although lower

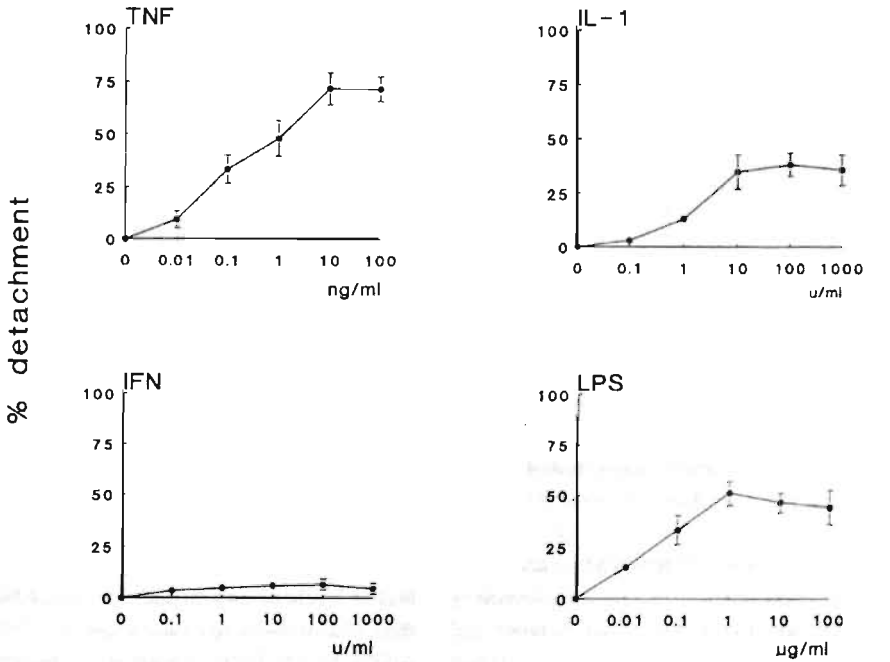

Fig. 3. Effects of TNF-α, IL-1α, IFN-γ, and LPS on neutrophil mediated HUVEC-detachment. Neutrophils (T/E = 1/50) were added together with the agents to the assay. Cells were coincubated for 3h at 37°C. A representative experiment out of series of 3 is shown. Data indicate mean ± SD of 4 measurements.

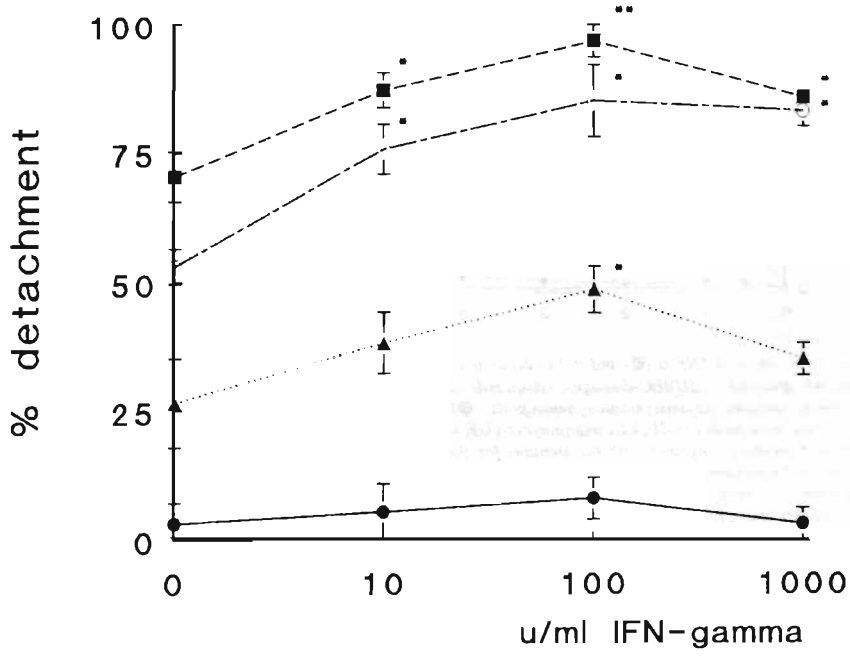

Fig. 4. *Effects of combined addition of cytokines on neutrophil mediated HUVEC-detachment. Neutrophils (T/E = 1/50) were added together with cytokines and incubated for 3h at 37°C.*
Different concentrations of IFN-γ were added alone (●), or in combination with 10ng/ml TNF-α (■), 10u/ml IL-1α (▲), or both TNF-α and IL-1α (O).
A representative experiment out of a series of 3 is shown. Data indicate mean ± SD of 4 measurements.
** $p<0.05$ and ** $p<0.01$ when compared to endothelial cell detachment under identical conditions, in absence of IFN-γ, as determined by the unpaired one-tailed Student's t-test.*

values were reached as with TNF-α. IFN-γ failed to cause a clear increase in endothelial cell damage. LPS induced about 50% endothelial cell detachment, when added at concentrations higher than 100ng/ml *(Fig. 3)*.

Since the cytokines IFN-γ, TNF-α and IL-1α are expected to be produced simultaneously in vivo, we studied the effect of combined administration of these cytokines. IFN-γ showed a moderate potentiating effect on IL-1α and/or TNF-α, when present in concentrations of 10, 100 or 1000 u/ml *(Fig. 4)*. Combined addition of 10ng/ml TNF-α and 100u/ml IFN-γ resulted in nearly complete disruption of the HUVEC monolayer. IL-1α and TNF-α did not prove to have a synergistic effect on endothelial cell damage *(Fig. 4)*. Combining TNF-α with LPS did not induce higher levels of endothelial cell detachment than TNF-α alone (data not shown).
Addition of heat-inactivated (10min at 100°C) TNF-α, IL-1α and IFN-γ, did not increase neutrophil mediated endothelial cell

Table 1. Effects of separate incubations with 10ng/ml TNF-α on neutrophil induced HUVEC detachment

	Exp. 1	Exp. 2	Exp. 3	Exp. 4
TNF-α coincubation[a)]	79 ± 9	63 ± 5	84 ± 5	81 ± 4
TNF-α preincubation of neutrophils[b)]	74 ± 2	66 ± 6	74 ± 9	75 ± 8
TNF-α preincubation of HUVEC[c)]	3 ± 2	-4 ± 4	9 ± 4	11 ± 6
„[c)] plus neutrophil preincubation[b)]	53 ± 4	71 ± 8	75 ±12	69 ± 7
„[c)] plus TNF-α coincubation[a)]	59 ± 5	n.t.	n.t.	n.t.

HUVEC detachment was measured after 3h incubation with neutrophils (T/E = 1/50) at 37°C. Data indicated mean ± S.D. of the percentage detached endothelial cells, of 4 measurements.
a) 10ng/ml TNF-α was present during the assay. b) Neutrophils were incubated with 10ng/ml TNF-α for 30min at 4°C, rinsed twice and added to the HUVEC-monolayers. c) TNF-α preincubation of HUVEC-monolayers was performed by adding TNF-α in a small volume to the HUVEC during the last 4h of culture with ^{51}Cr.

detachment (data not shown), indicating that the effects of the cytokine-preparations were not due to endotoxin contamination.

Characterization of TNF-α induced endothelial cell detachment.

To investigate whether TNF-α induces its effect mainly by a direct activation of neutrophils, or by modulating the endothelial cell monolayer, we stimulated either neutrophils or endothelial cells, before performing the endothelial cell damage assay under standard conditions (3h incubation, T/E=1/50). The results of 4 independent experiments are shown in *Table 1*.

Addition of neutrophils, preincubated with 10ng/ml TNF-α for 30min at 4°C, induced detachment levels similar to levels measured after coincubation of neutrophils and TNF-α during the assay. Next, the endothelial cells were incubated for 4h with 10ng/ml TNF-α to acquire maximal neutrophil-adhesiveness via the induction of ELAM-1 (4, 6).

Expression of ELAM-1 was confirmed in parallel-microtiter plates in an ELISA using ELAM-1 specific Mab as reported (27) (data not given). TNF-α preincubation of HUVEC increased neutrophil adhesion from 8-19% to 51-71%, as measured in a parallel adhesion assay, as described (29) (data not shown). Endothelial cell detachment, after incubation TNF-activated endothelium with unstimulated neutrophils for 3h, was not increased above background by this treatment *(Table 1)*. Moreover, TNF-activated endothelium did not appear to be more vulnerable to, or to trigger additional injury by TNF-α-activated neutrophils *(Table 1)*.

Since neutrophil mediated endothelial cell detachment in presence of TNF-α appeared to depend on direct activation of neutrophils by TNF-α, we investigated kinetics and conditions of neutrophil activation by TNF-α. Preincubation of neutrophils with 10ng/ml TNF-α at 37°C under non-adherent condi-

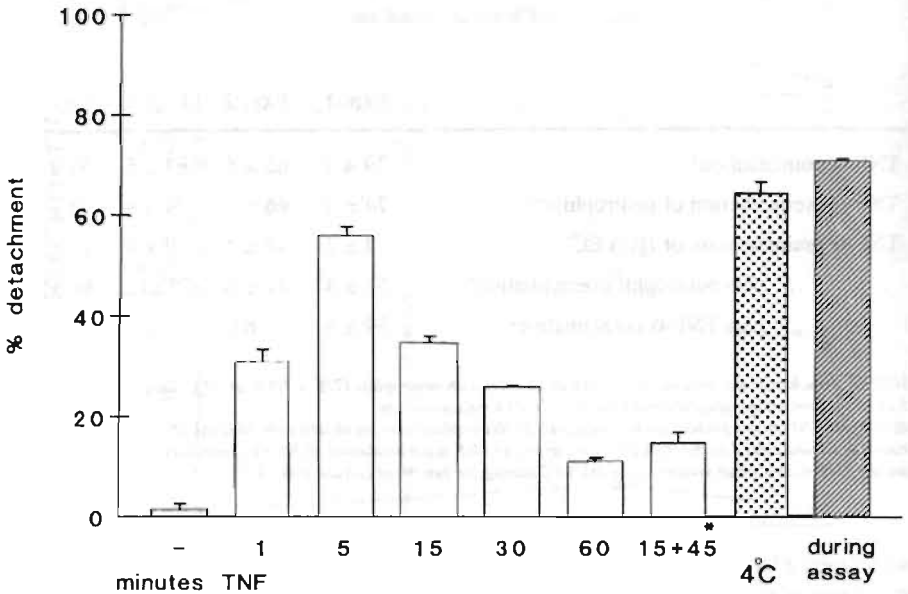

Fig. 5. Effect of neutrophil preincubation with TNF-α for different times at 37°C (open bars), compared to neutrophil preincubation for 60min at 4°C (dotted bar), and to TNF-α presence during the assay (hatched bar), on HUVEC-detachment. TNF-α was used at 10ng/ml. Neutrophil preincubation were performed as mentioned in Materials and methods. HUVEC-detachment was measured after 3h incubation with neutrophils (T/E = 1/50) at 37°C. A representative experiment out of a series of 4 is shown. Data indicate mean ± SD of 6 measurements.
** Neutrophils were incubated with TNF-α for 15 min at 37°C, rinsed, and incubated in absence of stimulus for another 45min at 37°C.*

tions, showed to induce a rapid activation, followed by a nearly complete deactivation of neutrophils, as measured by their capacity to induce subsequent endothelial cell detachment *(Fig. 5)*.

Maximal activation of neutrophils was reached after 5min, and was similar to activation induced by 60 min of preincubation with TNF-α at 4°C, or to activation of neutrophils by presence of TNF-α during the assay *(Fig. 5)*. Preincubation of neutrophils in absence of stimulus for 60min at 37°C did not diminish their response on TNF-α during the assay, as compared to neutrophils kept at 4°C for 60min (data not given).

The decrease in neutrophil activation, measured after prolonged preincubation with TNF-α at 37°C, did not depend on continuous presence of TNF-α, since 60min TNF-activated neutrophils showed a reduced capacity to detach endothelial cells, independent of whether they where continuously incubated with TNF-α, or rinsed after 15min and incubated further for 45min at 37°C in absence of TNF-α *(Fig. 5)*.

Discussion

In this study we demonstrated that the cytokines TNF-α and IL-1α, and LPS can induce endothelial cell detachment in presence of neutrophils. It appeared that also PMA induced high levels of endothelial cell detachment, which is in concordance with previous reports (30, 31). The cytokine IFN-γ did not induce significant endothelial cell detachment, but enhanced moderately TNF-α or IL-1α induced activation.

This enhancing effect of IFN-γ may explain the contribution of IFN-γ in the elicitation of the Shwartzman reaction and the concomitantly occurring endothelial damage, as described by Billiau et al. (32).

Recent reports described a s similar enhancing effect of IFN-γ on TNF-α and IL-1 induced ELAM-1 expression by endothelial cells, as well as on IL-6 release by endothelial cells (15, 33, 34).

We investigated the effect of 4 hour preincubation of endothelial cells with TNF-α on subsequent neutrophil mediated endothelial cell injury. Endothelial cell detachment, mediated by either untreated or TNF-activated neutrophils was not increased by this treatment, although these monolayers showed highly increased adhesiveness for neutrophils. Other data support the hypothesis that the function of endothelial responses on treatment with TNF-α, such as expression of adhesion molecules, is limited mainly to leukocyte-adhesion instead of triggering leukocytes to induce direct local damage.

A recent study by Mier et al. (35) showed that TNF-α preincubation of endothelium diminished LAK-cell induced endothelial cell lysis and endothelial cell monolayer permeability, although TNF-α preincubation of endothelial cells enhanced LAK-cell adhesion. Additionally, TNF-α preincubation of endothelium did not result in activation of neutrophils, placed on such endothelium, since no influence on elastase release or respiratory burst activity could be measured (36).

On the other hand, previous reports of Varani et al. (37) and Gibbs et al. (36) stated that respectively 4 to 18h preincubation of endothelium with TNF-α, increased their vulnerability to PMA or C5a activated neutrophils. The increase of neutrophil induced endothelial cell monolayer permeability induced by TNF-α pretreatment reported by Gibbs et al was, however, rather small, and only significant at higher TNF-α concentrations.

Results of Varani et al were obtained using rat pulmonary endothelial cells. In a later report of this group, they stated that such a TNF-α induced enhanced susceptibility for neutrophil-mediated damage was absent in endothelial cells derived from human umbilical veins (as we used here), and suggested that the two endothelial cell populations had a significant biological difference in their response to TNF-α (38).

We demonstrated in this study that the endothelial cell damage, induced by TNF-α in presence of neutrophils, depends on activation of neutrophils by TNF-α. Neutrophils can produce two groups of products, reactive oxygen metabolites and proteases, which bothe damage endothelial cells (28, 30, 39-41). TNF-α activation of neutrophils incites both production of large amounts of H_2O_2 and O_2-, and release of the content of primary and secondary granules of neutrophils (9-11), which thus seems in agreement with

a role for TNF-α in triggering neutrophils to induce endothelial cell damage. Under the conditions described in this report, endothelial cell lysis remained negligible and damage was restricted to detachment of the cells from the substratum.

Compared to other cell-populations, such as fibroblasts (37, 42) and kidney-cells (43), endothelial cells are relatively vulnerable to oxidant-injury, making endothelial cell damage an early pathophysiological phenomenon in syndromes involving free radical generation (44). H_2O_2 exposure to endothelial cell in vitro, can cause a number of time and concentration dependent changes, ranging from changes in intra-cellular free Ca^{++} and the release of PAF, to ATP breakdown, cell retraction, and lysis (42, 45, 46).

In such in vitro assays in which reactive oxygen species involving endothelial cell lysis is measured, complete culture media are usually replaced by balanced salt solutions, lacking serum. Serum presence is a prerequisite for normal endothelial cell growth and function (47, 48), and can protects against endothelial cell lysis (38). Besides the presence of 10% bovine calf serum in our assay-medium, the source of the endothelial cells used here might also have contributed to the absence of endothelial cell lysis in our set of experiments. HUVEC show, in contrast with rat pulmonary endothelial cells (38) and cow pulmonary endothelial cells (45), increased resistance to neutrophil mediated killing. This resistance has been contributed to increased activity of glutathione-peroxidase linked mechanisms to detoxify H_2O_2 in HUVEC (38, 45).

We showed that prolonged incubation of suspended neutrophils with TNF-α at 37°C, but not at 4°C, led to a reduction of endothelial cell detachment induced subsequently by these neutrophils. These results are in concordance with a recent report of Schleiffenbaum et al. (12), who demonstrated that prolonged incubation with either TNF-α, FMLP, C5a and the Calcium ionophore A12357 decreases TNF-α binding capacity on neutrophils and abrogates the production of H_2O_2 upon subsequent TNF-α exposure. Porteu et al. (49) demonstrated that such a decrease in TNF-α binding capacity is caused by shedding of the neutrophil TNF receptor, which occurs within 15 min after addition of a stimulus. No receptor-shedding was measured when neutrophil-stimulation was performed at 4°C and 16°C. These in vitro findings may reflect a mechanism which prevents excessive activation of circulating neutrophils in situations of systemic cytokine challenge. Especially local presence of TNF-α therefore could play a crucial role in endothelial cell damage seen in acute inflammatory syndromes, like multiple organ failure during septic shock, and acute allograft rejection.

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Chapter 5.2

FCγ RECEPTOR-MEDIATED ACTIVATION OF NEUTROPHIL H_2O_2-RELEASE AND OF NEUTROPHIL MEDIATED ENDOTHELIAL CELL DAMAGE BY A MONOCLONAL ANTIBODY AGAINST ELASTASE

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Summary

A mAb directed against human neutrophil elastase was tested for its ability to activate isolated human neutrophils. Neutrophils incubated with anti-elastase mAb released high amounts of H_2O_2, whilst isotype matched control mAb were ineffective. The amount of H_2O_2 release was similar to H_2O_2 release by TNF-activated neutrophils, and could not be increased by combining TNF and anti-elastase mAb. In parallel, neutrophil mediated injury of cultured human umbilical vein endothelial cell (HUVEC[3]) monolayers was measured. Anti-elastase mAb induced neutrophil mediated HUVEC detachment, although to a lesser extent than TNF. The mechanism of anti-elastase mAb induced neutrophil H_2O_2 release was investigated and was found to involve FcγRII, as evidenced by inhibiting anti-elastase mAb induced neutrophil H_2O_2-release by mAb reactive with Fcγ receptor II (FcγRII), and by showing that $F(ab')_2$ fragments of anti-elastase mAb failed to induce neutrophil activation. Furthermore, neutrophil H_2O_2-release induced by anti-elastase mAb could be inhibited by addition of mAb reactive with CD18 and with CD11b, suggesting simultaneous involvement of the β_2 integrin member CD11b/CD18 in anti-elastase mAb induced neutrophil activation.

These data demonstrate that elastase specific antibodies can trigger neutrophil respiratory burst activity and neutrophil mediated endothelial cell damage, via Fcγ receptor and β_2 integrin dependent mechanisms. Anti-elastase antibodies might play a role in the pathogenesis of tissue damage in clinical auto-immune syndromes with circulating elastase reactive antibodies, such as Wegener's granulomatosis.

Introduction

Presence of circulating autoantibodies against components of neutrophil cytoplasm (ANCA) is closely related to development of primary idiopathic systemic vasculitis syndromes including the granulomatosis (Wegener's granulomatosis and Churg-Strauss syndrome) and the polyarteritis group (classical polyarteritis nodosa, microscopic polyarteritis and its renal-limited variant idiopathic rapidly progressive glomerulonephritis) (reviewed in 1).

Two groups of anti-neutrophil cytoplasm antibodies have been defined, based upon their characteristic staining pattern after standard indirect immunofluorescence technique on methanol-fixed neutrophils: classical or cytoplasmic ANCA (cANCA) and the perinuclear ANCA (pANCA). Elastase, a poly-cationic serine protease found in the primary granules of neutrophils, has been identified as one of the pANCA targets, together with lactoferrin and the most predominant pANCA antigen, myeloperoxidase (2-6). Whether and how ANCA presence is related to the pathogenesis of systemic vasculitides, is unclear.

A number of recent data support the hypothesis that direct interaction of ANCA with neutrophils can trigger degranulation and activation of neutrophil respiratory burst activity, which might cause subsequent endothelial cell injury and trigger a cascade of pro-inflammatory and tissue-destructive events. *In vitro* activation of neutrophil respiratory burst activity and in some instances also degranulation has been demonstrated with isolated ANCA from patients with vasculitis (7), with mAb reactive with myeloperoxidase (7), and with a series of polyclonal antibody-preparations against ANCA antigens, including elastase (8). Furthermore, antibodies reactive with myeloperoxidase and proteinase 3 have been shown to increase neutrophil mediated endothelial cell damage (9, 10). The mechanism via which these autoantibodies trigger neutrophil activation is unclear.

In this *in vitro* study, we show that an anti-elastase mAb can induce the release of high amounts of H_2O_2 by isolated human neutrophils, and induce neutrophil mediated endothelial cell injury. Furthermore, we present evidence for involvement of FcγRII and of CD11b/CD18 in anti-elastase mAb induced neutrophil H_2O_2-release.

Materials and Methods

Antibody preparations and reagents.

Neutrophil reactive mAb were raised by standard hybridoma technique from spleens of mice immunized with human neutrophils. An anti-elastase mAb of the IgG_1 subclass, termed HM.4, was further characterized and used in this study.

Indirect immunofluorescence, performed using a modification of the method of van der Woude et al. (11), showed high and selective binding to ethanol-fixed human neutrophils with a characteristical pANCA immuno-fluorescence pattern. Selective reactivity of mAb HM.4 with human neutrophil elastase was confirmed by: 1) Binding to the elastase peak after chromatography of azurphilic granule extracts on an Orange A Matrex column, as discribed previously (12). 2) Binding to commercial elastase, but not to lactoferrin or myeloperoxidase. 3) Fluid phase inhibition of the binding of mAb HM.4 to fixed neutrophils with purified elas-

tase. HM.4 binding to commonly used human cells and cell-lines was investigated. No HM.4 binding to HUVEC and low HM.4 binding to lymphoid cells and monocytes was found.

For use in *in vitro* neutrophil activation assays, protein G purified antibody-preparations were used. $F(ab')_2$ fragments were prepared by pepsin (Immobilized Pepsin, Pierce, Rockford, IL) digestion according to the manufacturers instructions.

The following mAb were employed to identify receptor-involvement in anti-elastase mAb induced neutrophil activation: mAb IB4 (IgG_{2a}), reactive with CD18 (13), a gift of Dr. M. Daha, University Hospital Leiden, The Netherlands; mAb 60.3 (IgG_{2a}), reactive with CD18 (14), a gift of Dr. P.G. Beatty, Fred Hutchinson Cancer Research Center, Seattle, WA; mAb 904 (IgG_1) reactive with CD11b (15), a gift of Dr. J.D. Griffin, Dana Farber Cancer Institute, Boston, MA; mAb PDV11.2 (IgG_{2a}), reactive with CD11b (16), a gift of Dr. F. Koning, University Hospital Leiden, The Netherlands, mAb 44 (IgG_1), reactive with CD11b (17), a gift of Dr. N. Hogg, ICRF, London, UK; mAb TB-3 (IgG_{2a}), reactive with FcγRI (18), a gift of Dr. R. A. W. van Lier, CLB, Amsterdam, The Netherlands; mAb CIKM5 (IgG_1), reactive with FcγRII (19), a gift of Dr. G. Pilkington, Australia; mAb 3G8 (IgG_1), reactive with FcγRIII (20), a gift of Medarex, West Lebanon, NH, and mAb CLB/Fcgran1 (IgG_{2a}), reactive with FcγRIII (21), a gift of Dr. T. Huizinga, CLB, Amsterdam, The Netherlands. mAb 3.9 (IgG_1), reactive with CD11c (22), a gift of Dr. N. Hogg, ICRF, London, UK, and the non-specific mAb MOPC21 (IgG_1), kindly provided by Celltech (Slough, UK), were used as a control. Recombinant human (rh) TNF-α was kindly provided by BASF/Knoll AG, Ludwigshafen, FRG. PMA was purchased from Sigma, St. Louis, MO.

Cells

Neutrophils were prepared from buffy coats of donor blood kindly provided by the local blood bank, using standard techniques of buoyant density centrifugation on Lymphoprep (Nycomed, Oslo, Norway), followed by separation of neutrophils from erythrocytes by density gradient centrifugation on percoll (1.08 g/ml, Pharmacia, Uppsala, Sweden). Neutrophil preparations contained <5% contaminating cells, as evaluated by May Grunwald Giemsa staining.

HUVEC were obtained by collagenase treatment of the human umbilical vein and cultured in fibronectin-coated (fibronectin kindly provided by Dr. J. van Mourik, CLB, Amsterdam, The Netherlands) tissue culture flasks (Costar, Cambridge, MA) in culture medium.

Culture medium was composed of RPMI-1640 (Gibco Europe, Paisley, Scotland), supplemented with 10% heat-inactivated human serum, 10% heat-inactivated bovine calf serum (BCS) (Hyclone, Logan, UT), 50 μg/ml heparin (Sigma), 30 μg/ml endothelial growth supplement (Collaborative Research Incorporated, Bedford, MA) and antibiotics. Endothelial cells were characterized by their pavement-like monolayer morphology and by positive staining with the anti-E-selectin mAb ENA1 (23) after 4 h incubation with TNF-α.

Injury assay

^{51}Cr-release and detachment assays were performed as described before (24). In short, HUVEC (passage 3) were grown to confluence during 24 h in fibronectin-coated 96-well flat bottom microtiter plates (Costar). The last 16 h of this culture Chromium-51 as sodium [^{51}Cr]chromate was added to the wells (18 kBq/well, specific activity 11 GBq/µg Cr). Monolayers were carefully washed, and neutrophils (target-effector cell ratio 1:50) and reagents in RPMI-1640 supplemented with 10% BCS were added to the monolayer at a final volume of 200 µl/well.

Incubations were performed at 37°C for 3 h. Endothelial cell lysis was determined by measuring ^{51}Cr release in 100 µl cell-free supernatant medium, removed from each well with care not to disturb the monolayer. Next, 100 µl of fresh medium was added to the remaining 100 µl in each well. Detached endothelial cells were suspended by repeated careful pipetting, and 100 µl of this suspension was removed to determine detachment (these samples thus contained 50% of the amount of ^{51}Cr in the lysis samples, plus the ^{51}Cr present in the detached cells). Maximal ^{51}Cr content was determined in wells receiving 100 µl of 1 N NaOH.

All samples were counted in a gamma-spectrophotometer. Detachment values were calculated by subtracting half of the ^{51}Cr counts measured in the lysis samples drawn from the same wells, from the ^{51}Cr counts measured in the detachment samples, and corrected for non-specific ^{51}Cr content, by subtracting ^{51}Cr counts measured in detachment samples from wells without effector cells.

H_2O_2 measurement

A previously described colorimetric method to determine H_2O_2 production was used (24). In short, neutrophils, reagents and mAb were suspended/dissolved in RPMI-1640 supplemented with 10% BCS, antibiotics, 200 µg/ml phenol red (Sigma) and 8.5 u/ml horse radish peroxidase (type VI, Sigma). Neutrophils and reagents were placed in microtiter-plates coated with BCS at 2.5×10^5 cells/well to a final volume of 200 µl/well, and incubated for 3 h at 37°C. 50 µl 2M NaOH was added and OD was determined at 620 nm. The lower sensitivity of the assay was 1.6 µM H_2O_2.

Results

Anti-elastase mAb HM.4 induces neutrophil H_2O_2-release

Prolonged adhesion dependent neutrophil H_2O_2-release can be elicited by particles such as zymosan, and by cytokines such as TNF (24-26). Neutrophil binding murine mAb of the IgG_1, IgG_{2a}, or IgG_{2b} subclass, used to interfere with the function of neutrophil surface structures such as CD11a, CD11b, CD11c, CD18 do not incite prolonged neutrophil H_2O_2-release (24-27).

Furthermore, no difference between complete mAb, and $F(ab')_2$ fragments was evident in these inhibition studies (24). Additionally, we tested a panel of murine mAb reactive with the neutrophil surface structures L-selectin, CD14, FcγRI, FcγRII and FcγRIII which all failed to influence prolonged neutrophil H_2O_2-release (data not shown). In contrast, incubation of isolated human neutrophils with mAb HM.4, an anti-elastase mAb of the IgG_1 subclass, for 3 h at 37°C resulted in a concentration dependent increase of neutrophil H_2O_2-release *(Fig. 1)*,

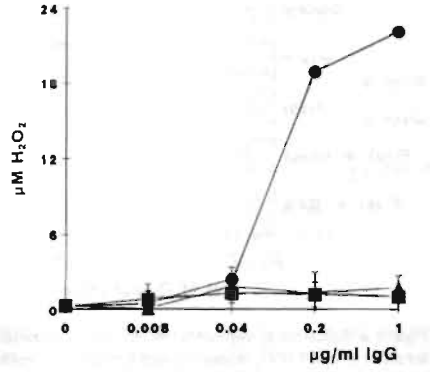

Figure 1. Influence of anti-elastase mAb HM.4 (●), anti-CD11c mAb 3.9 (▲) and the non-binding control mAb MOPC21 (■) on H_2O_2 release by isolated human neutrophils. Antibodies and cells were added simultaneously to BCS coated microtiterplates, and H_2O_2 release was measured after 3 h. Data indicate mean and SD of 4 replicates. If SD bars are not shown, they fall within the symbol.

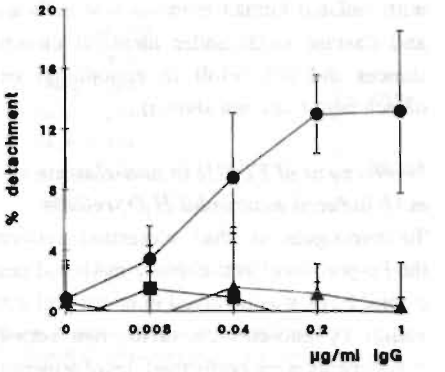

Figure 2. Influence of anti-elastase mAb HM.4 (●) anti-CD11c mAb 3.9 (▲) and the non-binding control mAb MOPC21 (■) on neutrophil mediated endothelial cell detachment. Neutrophils were added together with the mAb to HUVEC monolayers. Endothelial cell lysis and endothelial cell detachment were measured after 3 h of incubation at 37°C, as indicated in material and methods. Endothelial cell lysis remained consistently below 5% of the total number of HUVEC. Data indicate mean and SD of 4 measurements.

reaching maximal stimulation at 1 µg/ml. Two isotype-matched control mAb were tested in parallel, a non-specific mAb without neutrophil binding capacity, and a neutrophil-reactive anti-CD11c mAb. Both mAb's failed to induce neutrophil H_2O_2-release at identical Ig concentrations *(Fig. 1)*.

The anti-elastase mAb was found to be nearly equally potent in triggering neutrophil H_2O_2-release as the known neutrophil agonists TNF-α and PMA (1 µg/ml anti-elastase mAb HM.4: 22 ± 0.3 µM H_2O_2, 10 ng/ml TNF-α: 26 ± 0.6 µM H_2O_2, 50 ng/ml PMA: 28 ± 1.0 µM H_2O_2, measured in the same experiment after 3 h of coincubation).

Combined addition of 10 ng/ml TNF-α and 1 µg/ml anti-elastase mAb did not further increase neutrophil H_2O_2 release (26 ± 0.9 µM H_2O_2 in the same experiment).

Anti-elastase mAb HM.4 induces neutrophil mediated HUVEC detachment

^{51}Cr-labeled HUVEC monolayers were incubated with neutrophils (target : effector-cell ratio 1 : 50) and antibodies for 3 h at 37°C to investigate whether neutrophils could be activated to induce endothelial cell injury. Neutrophil incubation with anti-elastase mAb, but not with anti-CD11c or non-reactive control mAb, caused a concentration dependent increase of neutrophil mediated HUVEC detachment *(Fig. 2)*, but no endothelial cell lysis (data not shown).

The influence of anti-elastase mAb on neutrophil mediated endothelial cell injury was relatively small in comparison to 10 ng/ml TNF-α, which induced 86 ± 6% HUVEC detachment, without HUVEC lysis, in the

same experiment. Incubation of HUVEC with isolated human mononuclear cells and anti-elastase mAb under identical circumstances did not result in endothelial cell detachment (data not shown).

Involvement of FcγRII in anti-elastase mAb induced neutrophil H_2O_2-release

To investigate whether interaction between the Fc-portion of anti-elastase mAb and neutrophil FcγR was involved in neutrophil activation by anti-elastase mAb, two sets of experiments were performed. Involvement of the Fc-portion was investigated by testing $F(ab')_2$ fragments of anti-elastase mAb HM.4 for their capacity to induce neutrophil H_2O_2 release. Whilst 1 μg/ml intact anti-elastase mAb induced 40 μM H_2O_2 release after 3 h of incubation, equimolar concentrations of $F(ab')_2$ fragments of these anti-elastase mAb did not trigger neutrophil H_2O_2 release *(Fig. 3)*.

The loss of the capacity to activate neutrophils after pepsin-digestion of anti-elastase mAb was not due to a decrease in the affinity of the $F(ab')_2$ fragments for neutrophils, as evidenced by positive pANCA staining using these $F(ab')_2$ fragments (data not shown). Moreover, addition of an excess of elastase reactive $F(ab')_2$ fragments inhibited neutrophil activation by 0.1 μg/ml of intact anti-elastase mAb *(Fig. 3)*, confirming that the anti-elastase $F(ab')_2$ fragments do not have a decreased affinity to neutrophil bound elastase, and suggesting the involvement of saturatable antigen specific interactions between anti-elastase mAb and neutrophils in neutrophil activation by anti-elastase mAb.

To investigate whether cross-linking of neutrophil bound elastase to larger complexes

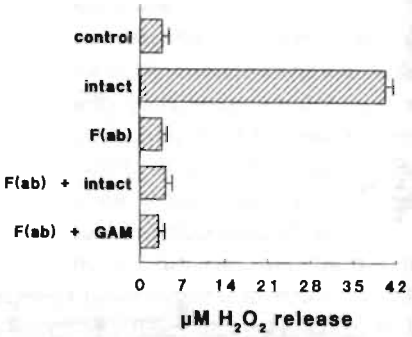

Figure 3. Influence of different combinations of antibodies on neutrophil H_2O_2 release: intact anti-elastase mAb, $F(ab')_2$ fragments of anti-elastase mAb, intact anti-elastase mAb plus $F(ab')_2$ fragments of anti-elastase mAb, and $F(ab')_2$ fragments of polyclonal goat anti-mouse antibodies after preincubation with $F(ab')_2$ fragments of anti-elastase mAb. Intact mAb were used at 1 μg/ml, all $F(ab')_2$ fragment prepartions were used at a concentration equimolar to 1 μg/ml intact mAb. Data indicate mean and SD of 4 replicates.

would be involved in anti-elastase mAb induced neutrophil activation, we tested whether Fc-fragment independent cross-linking of surface bound elastase by preincubation with anti-elastase $F(ab')_2$ fragments, and subsequent addition of goat-anti-mouse $F(ab')_2$ fragments would trigger neutrophil H_2O_2 release. No increase in neutrophil H_2O_2 release, however, was induced by these mAb fragments (Fig. 3), indicating that complexation of neutrophil membrane elastase itself is not sufficient to cause neutrophil activation.

To investigate the involvement of FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) in neutrophil activation by anti-elastase mAb, a number of FcγR reactive mAb known to prevent FcγR antibody interaction, were tested on their influence on anti-elastase mAb induced neutrophil H_2O_2-release. mAb TB-3 reactive with FcγRI and mAb

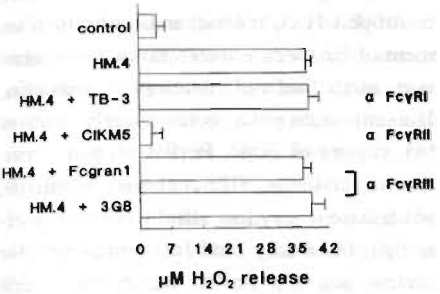

Figure 4. Influence of mAb reactive with FcγRI, FcγRII and FcγRIII on H_2O_2 release by neutrophils activated with anti-elastase mAb. Neutrophils were incubated for 3 h in presence of 0.1 μg/ml anti-elastase mAb and 5 μg/ml of the indicated anti-Fcγ receptor mAb, after which the released amount of H_2O_2 was determined. Data indicate mean and SD of 4 replicates.

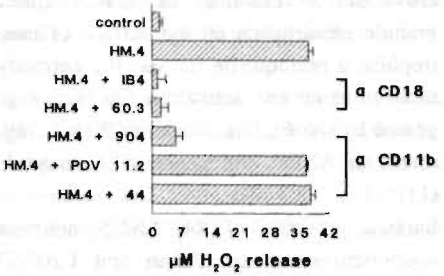

Figure 5. Influence of mAb reactive with CD11b and CD18 on H_2O_2 release by neutrophils activated with anti-elastase mAb. Neutrophils were incubated for 3 h in presence of 0.1 μg/ml anti-elastase mAb and 5 μg/ml of the indicated anti-CD11b/CD18 mAb, after which the released amount of H_2O_2 was determined. Data indicate mean and SD of 4 replicates.

CLB/Fcγgran1 and 3G8, reactive with FcRγIII failed to reduce anti-elastase mAb induced neutrophil H_2O_2 release, whilst mAb CIKM5 reactive with FcγRII completely prevented the mAb HM.4 induced increase in neutrophil H_2O_2 release (Fig. 4). None of the FcγR reactive mAb induced H_2O_2 release by unstimulated neutrophils (data not shown).

Involvement of the CD11b/CD18 integrin in anti-elastase antibody induced neutrophil activation

Substrate-contact mediated by the β_2 integrin dimer CD11b/CD18 facilitates neutrophil H_2O_2-release induced by zymosan (25) and TNF (24, 26). To further characterize the mechanism of anti-elastase antibody induced neutrophil activation, we investigated involvement of the β_2 integrin dimer in anti-elastase mAb induced neutrophil H_2O_2 release. Coincubation with two different CD18-reactive mAb, completely inhibited neutrophil H_2O_2 release induced by 0.1 μg/ml anti-elastase mAb. CD11b has two binding-sites, each engaged in the binding of distinct classes of ligands (27, 28). We observed a partial inhibition with mAb 904, reactive with the so-called LPS-binding region of CD11b (28), whereas two other anti-CD11b mAb (Fig. 5) and mAb reactive with CD11a and CD11c (data not shown) remained ineffective, suggesting involvement of specific substrate interactions via CD11b/CD18 in anti-elastase mAb induced neutrophil H_2O_2 release.

Discussion

In the present study we demonstrated that an elastase reactive mAb can specifically trigger neutrophil activation, as monitored via *in vitro* measurements of neutrophil H_2O_2-release and neutrophil mediated endothelial cell damage. Elastase is a serine protease which is present in abundant amounts in the primary granules of neutrophils, together with

other inflammatory proteins such as myeloperoxidase. Accessibility of ANCA-related granule constituents on the surface of neutrophils, a prerequisite for specific antibody induced neutrophil activation, has been suggested by the finding that even F(ab') fragments of ANCA can penetrate neutrophils (11).

Surface presence of the ANCA antigens myeloperoxidase, lactoferrin and CAP-57 has subsequently been demonstrated on activated neutrophils (8), thus facilitating FcγR independent binding of ANCA reactive with these proteins to activated neutrophils. We found strong binding of anti-elastase mAb HM.4 bound to both untreated and pre-activated neutrophils, and low or absent binding to lymphocytes, monocytes and endothelial cells (data not shown). This finding suggests presence of elastase on the neutrophil-surface, probably involving neutrophil elastase receptors, as described by Dwenger et al. (29). Neutrophils used in this study were isolated from buffy coats of donor blood using standard techniques of buoyant density gradient centrifugation, which are known to induce changes in surface expression of several proteins (30, 31). Wether neutrophils constitutively express elastase, or whether neutrophils become activated to some degree during their procurement and isolation, allowing translocation of elastase from their intracellular stores to the cell-surface, remains to be elucidated. We therefore can not exclude that anti-elastase mAb induced neutrophil activation might, as suggested for neutrophil activation by other ANCA (7, 8), require previous priming of neutrophils.

Elastase reactive $F(ab')_2$ fragments were, even after cross-linking with anti-mouse IgG $F(ab')_2$ fragments, not sufficient to induce neutrophil H_2O_2 release, indicating involvement of Fc-specific interactions in anti-elastase mAb induced neutrophil activation. Human neutrophils constitutively express two classes of FcγR, FcγRII (A and C isoforms) and the GPI-anchored FcγRIIIB, witch have a very low affinity for monomeric IgG, but avidly bind IgG complexes (for review, see 32). FcγRI, which has a high affinity for monomeric IgG, can be expressed on neutrophils after activation (32). Multivalent ligation of either FcγRII or FcγRIIIB, by immune-complexes, IgG coated particles or heat aggregated IgG, or by cross-linking FcγR specific mAb with a secondary antibody, induces cellular activation (32). Divalent ligation of FcγRII, as performed in this study by incubating neutrophils with mAb CIKM5, does not activate neutrophils. mFcγRII is thought to be primarily responsible for IgG-mediated activation, while FcγRIIIB serves as a trap to hold IgG-coated particles in place on the neutrophil surface, thereby enhancing contact with FcγRII and facilitating cell activation in a cooperative manner (33, 34). We found that a mAb reactive with FcγRII inhibited anti-elastase mAb induced neutrophil activation. FcγRII thus seems to play a role in anti-elastase mAb induced neutrophil activation, probably by direct interaction with the Fc-portion of anti-elastase mAb captured on the neutrophil surface. Another option would be that FcγRII functions as a signal-transducing molecule after association with FcγRIII molecules complexed with Ig (30, 34), but the absence of a reduction in neutrophil H_2O_2-release after addition of anti-FcγIII mAb makes critical involvement of this receptor unlikely.

Neutrophil activation by mAb reactive with

surface antigens is a rare finding. Myeloid-related mAb submitted to the Fourth Workshop on human leucocyte differentiation antigens were tested by Sunder-Plassmann et al (35) on their influence on neutrophil respiratory burst activity. Only five out of 165 tested mAb were found to increase neutrophil respiratory burst activity induced by opsonized zymosan. Most studies to define the characteristics of antigens which allow antibody-binding induced FcγR mediated activation have been performed using monocytes and platelets. In these studies, no relation was found between the number of cell-bound mAb and the likelihood of activation; an activating mAb against a surface protein having about 1,000 copies per cell has been described (36, 37). Cellular activation appears to depend mainly on the nature of the ligand, although differences between mAb reactive with the same receptor have also been described (35-37). Surface-mobility of the antigenic structure is thought to be critical, since this would allow association with and cross-linking of FcγR, which would elicit cellular activation (36).

Otherwise, ligand dependent FcγR 'perturbation' instead of cross-linking has been hypothesized to be the eliciting event for cellular activation (37). The elastase receptor on neutrophils has been poorly defined. Whether anti-elastase mAb induced neutrophil activation involves specific characteristics of an elastase-binding site on neutrophils or whether membrane-elastase simply acts as a mobile surface-ligand for specific IgG, thus facilitating FcγRII cross-linking awaits further study. Other factors could also be relevant, such as the proteolytic activity of elastase itself, which might have a role in FcγRII activation, and the relative abundance of binding sites for anti-elastase antibodies in comparison to the anti-CD11c which was used as a control mAb in this study.

Neutrophils can respond to stimulation with FMLP or PMA with rapid, adherence-independent H_2O_2-release (38, 39). Neutrophils stimulated with TNF, GM-CSF or LPS do not show rapid H_2O_2-release. In adequate substrate and medium-conditions, these neutrophils show progressive attachment and flattening, mediated by CD11b/CD18-substrate interactions and shedding of the negatively charged sialoprotein CD43, which enables subsequent massive H_2O_2-release (24, 26, 39-43). We demonstrated in this study that anti-elastase mAb induced neutrophil H_2O_2-release can be inhibited by mAb reactive with CD11b/CD18, suggesting that anti-elastase mAb induced neutrophil respiratory burst activation mimics the activation of neutrophil respiratory burst by TNF, GM-CSF and LPS concerning the need for substrate-interaction.

The absence of additive effects of TNF and anti-elastase mAb is congruent with this idea. TNF is, however, more potent than anti-elastase mAb in inducing neutrophil mediated endothelial cell detachment. Since direct effects of TNF on endothelial cells do not contribute to endothelial cell detachment in our model (44), TNF-induced and anti-elastase mAb induced neutrophil activation are partly discongruent.

In conclusion, we demonstrated that mAb directed against elastase can specifically induce neutrophil toxicity in vitro. On the basis of our data, we suggest that anti-elastase mAb bind to elastase on the neutrophil surface, enabling interaction between the

IgG Fc-portion and neutrophil FcγRII, leading to cellular activation. This activation subsequently induces CD11b/CD18 dependent release of reactive oxygen species.

This mechanism might provide a link between the presence of anti-elastase antibodies and the inflammatory tissue destruction found in patients with Wegener's granulomatosis and other forms of systemic autoimmune vasculitis.

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Chapter 5.3

INVOLVEMENT OF THE CD11B/CD18 INTEGRIN, BUT NOT OF THE ENDOTHELIAL CELL ADHESION MOLECULES ELAM-1 AND ICAM-1 IN TNF-α INDUCED NEUTROPHIL TOXICITY

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Abstract

TNF-α can incite neutrophil mediated endothelial cell damage, and neutrophil H_2O_2 release. Both effects require adherent neutrophils. Using specific mAb, we showed in this in vitro study that the CD18 β_2 chain and the CD11b α_M chain of the CD11/CD18 integrin heterodimer have a major role in both TNF-α induced neutrophil mediated detachment of human umbilical vein endothelial cells (HUVEC) and H_2O_2 release by TNF-α activated human neutrophils. In contrast to anti CD18 mAb, which consistently prevented neutrophil activation, anti CD11a mAb and two out of three anti CD11b mAb did not reduce endothelial cell detachment and neutrophil H_2O_2 release, although they decreased neutrophil adhesion to HUVEC. mAb 904, directed against the bacterial lipopolysaccharide (LPS) binding region of CD11b, reduced endothelial cell detachment for about 40% and neutrophil H_2O_2 release for more than 50%, demonstrating that CD11b/CD18 is engaged in TNF-induced neutrophil activation. Dependence on CD11b/CD18 could not be overcome by CD18 independent anchoring of neutrophils via PHA. Additionally, neither induction of increased expression of the endothelial cell adhesion molecules ICAM-1 and ELAM-1, nor subsequent addition of specific mAb, influenced endothelial cell injury or H_2O_2 release by TNF-activated neutrophils. Interaction with ICAM-1 and ELAM-1 therefore appears not to induce additional activation of TNF-stimulated neutrophils.

These studies suggest that a specific, CD11b/CD18 mediated signal, instead of adherence only, triggers toxicity of TNF-activated neutrophils.

Introduction

Neutrophil mediated inflammatory responses depend on adherence to endothelium, migration into an inflammatory site and on the release of toxic products by neutrophils. TNF-α influences these processes, by acting both on endothelium and on neutrophils. On endothelial cell monolayers TNF-α induces enhanced and *de novo* expression of adhesion molecules for neutrophils, such as ELAM-1 and ICAM-1 (1-4).

Neutrophil transmigration occurs rapidly after adherence to TNF-α activated endothelium (5, 6). Neither increased monolayer permeability (5, 6), nor endothelial cell detachment (7) is provoked during this event. In contrast, TNF-α activated neutrophils show only a transient increase in adhesiveness, mediated by the CD11/CD18 complex, whilst subsequent migration through endothelial cell monolayers is not induced (8, 9). TNF-α triggers respiratory burst activity and the release of the content of primary and secondary granules by adherent neutrophils (10-12), which can lead to endothelial cell damage in vitro (7).

The effect of neutrophil-activation by TNF-α depends on adherence; TNF-α induces only minimal degranulation and respiratory burst activity of neutrophils in suspension, or neutrophils adherent to uncoated plastic surfaces, whereas neutrophils adherent to protein coated plastic or endothelial cell monolayers produce large amounts of reactive oxygen species upon TNF-α exposure (10-15).

Receptor mediated interaction with the substratum therefore might function as an important second trigger.

Several membrane structures contributing to the adherence of neutrophils to endothelium have been revealed during the last decennium. In unstimulated conditions, interaction between the integrin member CD11a/CD18 (LFA-1) on neutrophils and ICAM-1 on endothelial cells is the main adherence pathway. Neutrophil activation induces increased expression and adhesive capacities of CD11b/CD18 (Mac-1, complement receptor 3), which results in a 50% share of CD11b in adhesion (9, 16). Additionally, endothelial cell activation by stimuli such as LPS, IL-1, TNF-α and phorbol esthers results in a slow increase in ICAM-1 expression (3, 4) and in *de novo* expression of ELAM-1 (1, 2), a representative of the LEC-CAM family of adhesion molecules (17). ELAM-1 adhesion involves sialyl Lewis X containing structures as a ligand (18-20), and results in highly increased, venous shear stress resistent adhesion of neutrophils (21). In spite of the detailed knowledge of the role of these membrane molecules in adhesion, little is known on their function in inter-cellular communication.

Previously we showed that considerable endothelial cell detachment, but no endothelial cell lysis, can be measured after addition of TNF-α activated neutrophils to cultured $HUVEC^3$ monolayers (7). In this study, we investigated the role of the neutrophil CD11/CD18 hetero-dimer and of ELAM-1 and ICAM-1, in TNF-α induced neutrophil toxicity. Using in vitro measurements of neutrophil induced HUVEC detachment, and neutrophil H_2O_2 release, evidence was obtained for a role of CD11b/CD18, other than mediating neutrophil adherence, in these processes.

Materials and Methods

Reagents

Recombinant human TNF-α was kindly provided by BASF/Knoll Ag. (Ludwigshafen, FRG). PMA was purchased from Sigma (St. Louis, MO), PHA (HA15) was purchased from Wellcome (Beckenham, Kent, UK).

Monoclonal antibodies

mAb ENA1 and ENA2 (both IgG1) were obtained by immunizing mice with IL-1 & TNF-α treated HUVEC, and react with different epitopes on ELAM-1 (2, 22). RR1/1 (IgG1), reactive with ICAM-1 (23), was a kind gift of Dr. Rothlein (Boehringer Ingelheim, Ridgefield, CT). mAb MOPC 21 (IgG1), a non specific control antibody, was kindly provided by Celltech (Slough, UK). mAb 60.3 (IgG2a), reactive with CD18 (24) was a kind gift of Dr. P.G. Beatty (Fred Hutchinson Cancer Research Center, Seattle, WA). mAb IB4 (IgG2a), reactive with CD18 (25), was a generous gift of Dr. M. Daha, University Hospital Leiden, The Netherlands. mAb CLB-LFA 1/1 (IgG1), reactive with CD18 (26) and CLB-LFA 1/2 (IgG2), reactive with CD11a (27) were kindly given by Dr. R.A.W. van Lier, CLB, Amsterdam, The Netherlands. mAb 25.31 (IgG1), reactive with CD11a (28), was generously provided by Dr. C. Mawas, INSERM, Marseille, France. mAb 44 (IgG1), reactive with CD11b (29) and mAb 3.9 (30), reactive with CD11c were kindly provided by Dr. N. Hogg, ICRF, London, UK. mAb PDV11.2 (IgG2a) reactive with CD11b (31) was a gift of Dr. F. Koning, University Hospital Leiden, The Netherlands. mAb 904, (IgG1) reactive with CD11b (32) was a generous gift of Dr. J.D. Griffin, Dana Farber Cancer Institute, Boston, MA. F(ab')$_2$ fragments were prepared by pepsin digestion (Immobilized Pepsin, Pierce, Rockford, IL) as described (2).

Cells

HUVEC were obtained by collagenase treatment of the human umbilical vein. The cells were cultured in fibronectin-coated (fibronectin kindly provided by Dr. J. van Mourik, CLB, Amsterdam, The Netherlands) tissue culture flasks (Costar, Cambridge, MA) in RPMI-1640 (Gibco Europe, Paisley, Scotland), supplemented with 10% heat-inactivated human serum, 10% heat-inactivated bovine calf serum (BCS) (Hyclone, Logan, UT), 50μg/ml heparin (Sigma), 30μg/ml endothelial growth supplement (Collaborative Research Incorporated, Bedford, MA) and antibiotics.

Endothelial cells were characterized by their pavement-like monolayer morphology and positive staining with mAb hec7 directed against PECAM-1 (33), generously provided by Dr. W.A. Muller, Rockefeller University, NY, and with the anti ELAM-1 mAb ENA1 and ENA2 after 4h incubation with TNF-α. Neutrophils were prepared from buffy coats of donor blood kindly provided by the local blood bank, using standard techniques of buoyant density centrifugation on Lymphoprep (Nycomed, Oslo, Norway), followed by separation of neutrophils from erythrocytes by density gradient centrifugation on percoll (1.08g/ml, Pharmacia, Uppsala, Sweden). Neutrophil preparations contained <5% contaminating cells, as evaluated by May Grunwald Giemsa staining.

Injury assay.

^{51}Cr-release and detachment assays were performed essentially as described by Diener et

al. (34). HUVEC (passage 3) were grown to confluence during 24h in fibronectin-coated 96-well, flat bottom, microtiter plates (Costar). Final plating density was ± $2x10^4$cells/well (well-surface: $0.32cm^2$). The last 16h of this culture Chromium-51 as sodium [^{51}Cr]chromate was added to the wells (18kBq/well, specific activity 11GBq/µg Cr).

At the beginning of the assay monolayers were carefully washed by five successive exchanges of 200µl/well RPMI-1640 1%BCS. Neutrophils and reagents in RPMI-1640 supplemented with 10%BCS were added to the monolayer as indicated, at a final volume of 200µl/well, and incubations were performed at 37°C for 3h, unless mentioned otherwise. Endothelial cell lysis was determined by measuring ^{51}Cr release in 100µl cell-free supernatant medium, removed from each well with care not to disturb the monolayer. Next, 100µl of fresh medium was added to the remaining 100µl in each well.

Detached endothelial cells were suspended by repeated careful pipetting, and 100µl of this suspension was removed to determine detachment (these samples thus contained 50% of the amount of ^{51}Cr in the lysis samples, plus the ^{51}Cr present in the detached cells in the sample). Control monolayers remained visually confluent after performing this procedure. Maximal ^{51}Cr content was determined in wells receiving 100µl of 1N NaOH, and reached about 10^5dpm. All samples were counted in a gamma-spectrophotometer. Detachment values were calculated by subtracting half of the ^{51}Cr counts measured in the lysis samples drawn from the same wells, from the ^{51}Cr counts measured in the detachment samples. Furthermore, measurements of endothelial cell lysis and endothelial cell detachment were corrected for non-specific ^{51}Cr content, by subtracting ^{51}Cr counts measured in lysis/detachment samples from wells without effector cells (in these wells spontaneous ^{51}Cr release and endothelial cell detachment ranged from 5 to 10% of total ^{51}Cr content).

Adherence assay

A visual adherence assay, essentially as described by C.W. Smith et al. (35) was utilized. Sterile microscope-slides were gelatin-coated by incubation with 0.75% gelatin (Difco, Detroit, MI) in PBS for 15min at room temperature (RT), fixed with 0.5% glutaraldehyde (Merck, Darmstadt, FRG) in PBS for 10min at RT, dip-rinsed 3 times in PBS, and air-dried. Slides were seeded with HUVEC one day prior to the experiment. Slides with visually confluent HUVEC monolayers were placed in specially constructed adherence chambers.

The chambers consisted of two metal clamps holding an uncoated and a HUVEC coated microscope slide, separated by two rubber O-rings (∅ 25mm). Within these two closed compartments neutrophils could be observed, using an inverted microscope with phase contrast optics. Neutrophils were pretreated for 30min with 10ng/ml TNF-α in RPMI-1640 supplemented with 10%BCS and antibiotics at 4°C in polypropylene tubes, and washed twice with ice-cold medium, or handled identically without TNF-α addition.

Subsequently, neutrophils were suspended in RPMI-1640 supplemented with 10%BCS and antibiotics at $5x10^4$cells/ml and further incubated with mAb (5µg/ml) for 5min at 37°C. Thereafter, 800µl neutrophil suspen-

sion was added to the chambers and allowed to settle onto the monolayer for a period of 10min at 37°C. The number of neutrophils present on the monolayer was determined by counting adherent neutrophils in 4 microscopic fields (x40 objective), and the chambers were inverted for another 10min at 37°C.

In the same fields, the percentage of cells remaining adherent on the monolayer was determined. In the results, the average of these 4 fields is expressed as percentage adherence.

H_2O_2 measurements

A colorimetric method was used to determine H_2O_2 production (36). HUVEC coated microtiter-plates, identical to the plates used in the injury assays, and microtiterplates coated with 50% BCS in RPMI-1640 for 60min at RT, were used. Neutrophils and agents were suspended/dissolved in RPMI-1640 supplemented with 10%BCS, antibiotics, 200µg/ml phenol red (Sigma) and 8.5u/ml Horse radish peroxidase (type VI, Sigma).

Neutrophils were preincubated with 10ng/ml TNF-α for 30min at 4°C, rinsed twice, and added to the wells at $2.5x10^5$cells/well, together with mAb (at a final concentration of 5µg/ml), to a final volume of 200µl/well, and incubated for 3h at 37°C. A standard H_2O_2 dilution range (highest concentration 100µM) was used.

Samples (150µl/well) were carefully removed at the end of the incubation period and OD was determined at 620nm after addition of 50µl 2M NaOH. The lower sensitivity of the assay was 1.6µM H_2O_2.

Detection of endothelial cell-surface antigens

HUVEC coated microtiter-plates, identical to the plates used in the injury assay, were washed and fixed with 0.05% glutaraldehyde for 10 min at RT. ELAM-1 and ICAM-1 expression were determined in ELISA's, using the ELAM-1 specific mAb ENA1, and the ICAM-1 specific mAb RR1/1, followed by peroxidase conjugated goat anti-rabbit IgG. O-Phenylene-diamine (Sigma) was added as a substrate and photospectometry was performed with a Microtiter ELISA reader at 492nm.

Results

Effect of anti CD11 and anti CD18 mAb on TNF-α induced neutrophil mediated endothelial cell detachment

To assess the potential role of the α and β chain of the β_2 integrin members CD11a/CD18, CD11b/CD18 and CD11c/CD18 in endothelial cell detachment induced by TNF-preincubated neutrophils, the influence of mAb directed against these molecules was tested. The effect of these mAb on adherence of TNF-α pretreated neutrophils to HUVEC was determined in parallel experiments.

mAb IB4, mAb 60.3 and mAb CLB LFA 1/1, all reactive with CD18 and added in concentrations adequate to reduce adherence of activated neutrophils to endothelium for more than 50% *(Fig. 1)*, nearly completely prevented endothelial cell detachment induced by neutrophils preincubated with 10ng/ml TNF-α for 30min at 4°C. mAb directed against CD11a, and two out of three mAb directed against CD11b did not influence endothelial cell detachment, although significant inhibition of adherence (ranging from 30 to 50%) was demonstrated in parallel

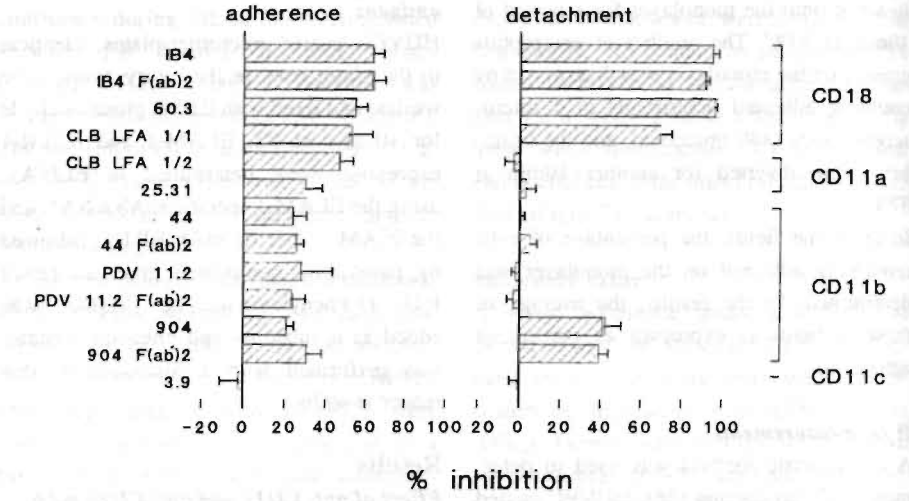


Figure 1. Influence of anti CD11/CD18 mAb on adherence of TNF-α stimulated neutrophils to endothelium (left graph), and detachment of endothelium by TNF-α stimulated neutrophils (right graph). Neutrophils were preincubated with 10ng/ml TNF-α for 30min at 4°C, and used in both the adherence and injury assays as mentioned in Materials and Methods, resulting in 19% neutrophil adherence and 62% HUVEC detachment. Data represent means and SE of the mAb induced inhibition in adherence and detachment, compared to adherence and detachment by TNF-α activated neutrophils in absence of mAb, from 4 separate experiments.

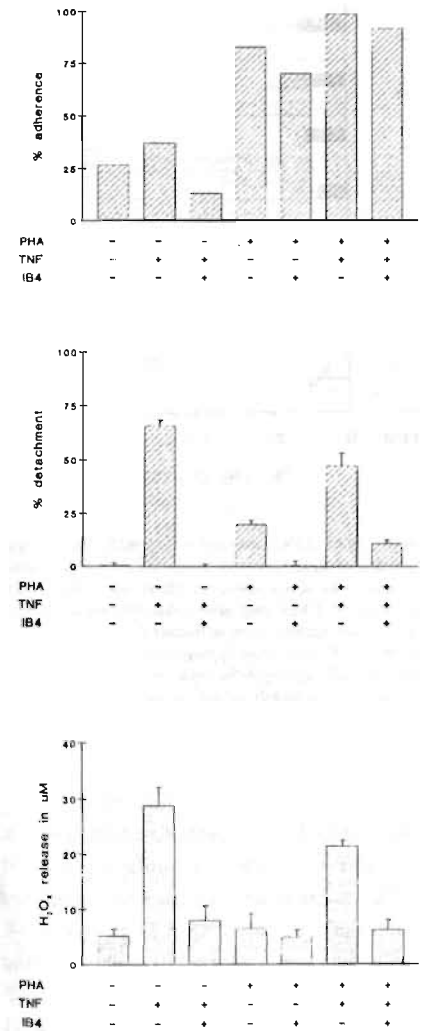
experiments ($p<0.05$ for each mAb, as compared with adherence in absence of mAb by the unpaired, two tailed Student's *t*-test). mAb 904, reactive with the LPS-binding region of CD11b (32, 37) induced a significant, 40% reduction in endothelial cell detachment ($p<0.01$ as compared with detachment in absence of mAb by the unpaired, two tailed Student's *t*-test).

$F(ab')_2$ fragments of al CD11b reactive mAb and of mAb IB4 directed against CD18 were also tested, and found to have similar influences on TNF-α induced neutrophil mediated endothelial cell detachment *(Fig. 1)*. Finally, the role of CD11c in endothelial cell detachment was investigated by using mAb 3.9 directed against CD11c. Neither a reduction in detachment nor a reduction in adhesion, induced by this mAb, was found.

MOPC 21 was used as a control mAb, and did affect neither detachment nor adhesion of neutrophils to endothelium (data not shown). The influence of anti CD18 and anti CD11a mAb on endothelial cell detachment was not specific for endothelium cultured on fibronectin-coated plastic, since culture of endothelium on gelatin-coated plastic instead of fibronectin-coated plastic did not markedly affect detachment values (data not shown).

Effect of PHA induced neutrophil-adherence on endothelial cell detachment by anti CD18 treated neutrophils

To answer whether the above described inhibition of neutrophil detachment by anti CD18 mAb depended on the decrease in



adhesion caused by these mAb, we investigated whether CD18 independent adherence could restore endothelial cell detachment by TNF-α activated and anti CD18 treated neutrophils. Addition of the multivalent lectin PHA was employed to induce CD18 independent anchoring of neutrophils to the endothelium. PHA induced high levels of adherence of both TNF-α pretreated and untreated neutrophils, without increasing CD18 dependent neutrophil adhesiveness *(Fig. 2A)*. A minor but reproducible increase in detachment of endothelial cells in presence of non-activated neutrophils was induced by PHA. IB4 preincubation of neutrophils inhibited endothelial cell detachment in presence of PHA by unstimulated as well as TNF-α stimulated neutrophils *(Fig. 2B)*.

The influence of ELAM-1 and ICAM-1 on neutrophil-activation

Expression of ELAM-1 and ICAM-1 after TNF-stimulation of HUVEC, in conditions identically to HUVEC used for the detachment and H_2O_2-release assays, was assessed by ELISA as mentioned in Materials and Methods. ELAM-1 expression was found to be below the detection level of the assay in unstimulated wells, and was 1.84 (A at 492nm) after 4h, 1.87 after 6h, and 1.54 after 8h of activation.

The time-interval between 4 and 7 hours after beginning of TNF-activation was therefore considered to be the most appropriate time to study the influence of ELAM-1 expression on neutrophil activation. ICAM-1

Figure 2. *Influence of PHA on adherence (A), HUVEC detachment (B) and H_2O_2-release (C). Neutrophils were subjected to none, one, or both of the following: preincubation with 10ng/ml TNF-α for 30min at 4°C, and preincubation with 5μg/ml IB4 for 30min at 4°C. Subsequently, neutrophils were added together with PHA (final concentration 1%) to both assays. Measurements were performed as described in Materials and Methods. Values are mean and SD of 4 measurements. A representative experiment out of a series of 3 is shown.*

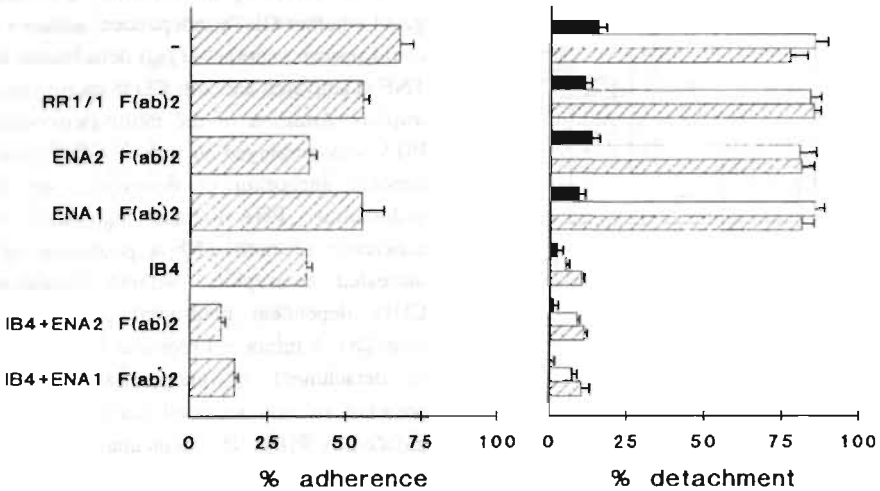


Figure 3. *Influence of anti ICAM-1 mAb (RR1/1), anti ELAM-1 mAb (ENA1, ENA2) and anti CD18 mAb (IB4) on neutrophil adherence to endothelium (left graph), and neutrophil mediated detachment of endothelium (right graph). Detachment was measured after HUVEC activation (filled bars), neutrophil activation (open bars) and both HUVEC and neutrophil activation (hatched bars). Adherence was measured after HUVEC and neutrophil activation. HUVEC were activated by incubation with 10ng/ml TNF-α for 4h at 37°C. Neutrophils were activated by incubation with 10ng/ml TNF-α for 30min at 4°C. Monolayers were incubated for 5min at RT with F(ab')$_2$ fragments of the anti ELAM-1 and ICAM-1 mAb directly before adding neutrophils plus the intact anti CD18 mAb. Adherence and detachment assays were performed as described in Materials and Methods. Unstimulated neutrophils added to unstimulated HUVEC showed 18±4% adherence and induced 4±4% HUVEC detachment in absence of mAb. Data given are mean and SE of 8 measurements from two independent experiments.*

expression was increased from 0.18 at t=0 to 0.92 after 4h and 1.14 after 18h of TNF-α stimulation. Neutrophil adhesion was increased from 18% to 62% by 4h TNF-α stimulation of HUVEC.

Up to 43% of this adherence was inhibited by anti ELAM-1 mAb, whilst a smaller but significant reduction was induced by F(ab')$_2$ fragments of RR1/1 directed against ICAM 1 *(Fig. 3 left graph,* $p<0.05$ as compared with adherence in absence of mAb by the unpaired, two tailed Student's *t*-test).

Substantial neutrophil mediated detachment of TNF-α activated endothelial cells was only seen after neutrophil activation by TNF-α, and was not further increased by previous activation of the endothelial cells *(Fig. 3 right graph).* Detachment of endothelial cells from TNF-α activated monolayers induced by TNF-α-pretreated neutrophils could only be inhibited by the anti CD18 mAb IB4. F(ab')$_2$ fragments of the ELAM-1 specific mAb ENA1 and ENA2, and F(ab')$_2$ fragments of the ICAM-1 specific mAb RR1/1 failed to induce a significant reduction in endothelial cell detachment *(Fig. 3 right graph).*

These experiments were repeated using neutrophils which were activated by coincubation with TNF during the detachment assay. Again we found no influence on endothelial cell detachment by 4h TNF activation of

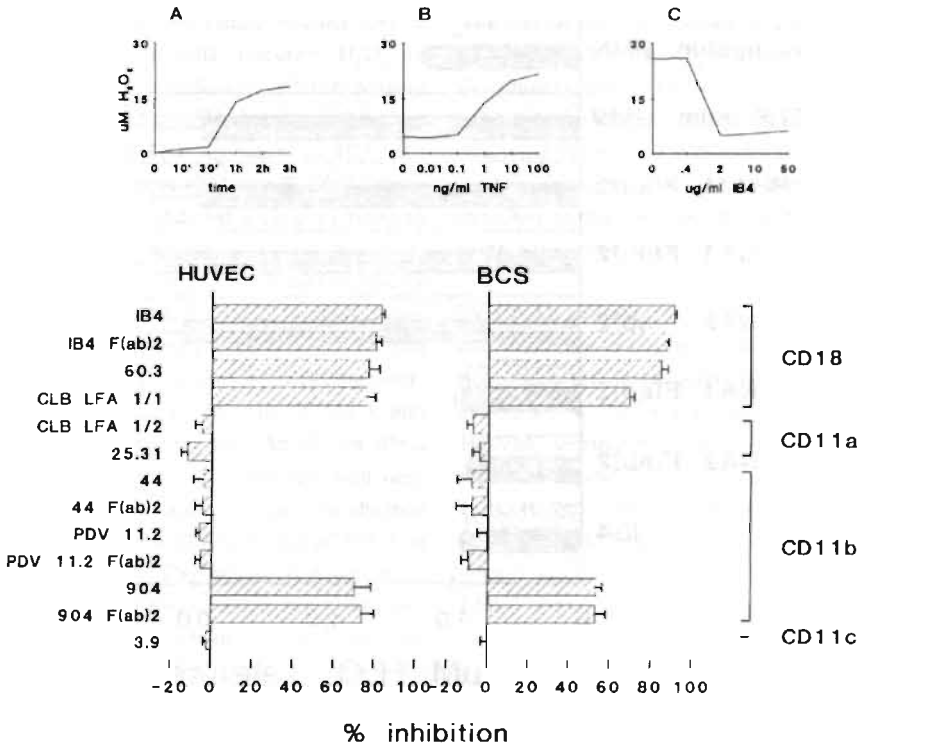


Figure 4. Influence of anti CD11/CD18 mAb on H_2O_2 release by TNF-α stimulated neutrophils placed on either endothelium (left graph) or BCS coated plastic (right graph). ***Insets:*** *(A) H_2O_2 release in time by 10ng/ml TNF-α stimulated neutrophils on endothelium. (B) H_2O_2 release by neutrophils, preincubated with different concentrations of TNF-α, on endothelium, after 3h of culture. (C) Effect of different concentrations of mAb IB4, directed against CD18 on H_2O_2 release by 10ng/ml TNF-α stimulated neutrophils on endothelium.* ***Main Figure:*** *Neutrophils were preincubated with 10ng/ml TNF-α for 30min at 4°C, and added to 96-well microtiter plates which had been seeded with HUVEC or coated with BCS. mAb were added together with the neutrophils to a final concentration of 5μg/ml each. H_2O_2 release was measured after 3h at 37°C, and reached 37μM on HUVEC and 58μM on BCS coated plastic in absence of mAb. Data represent means and SD of the mAb induced inhibition in H_2O_2-release, compared to H_2O_2-release by TNF-α activated neutrophils in absence of mAb, from 4 measurements. A representative experiment out of a series of 3 is shown.*

HUVEC, nor by subsequent addition of anti ELAM-1 and anti ICAM-1 antibody-fragments (data not shown). Also, prolonged TNF-activation of HUVEC to further increase ICAM-1 expression did not result in HUVEC activation dependent alterations in the amount of neutrophil mediated endothelial cell detachment.

Receptor involvement in TNF-α induced H_2O_2 release

To further asses whether CD11/CD18, ELAM-1 and ICAM-1 receptors were involved in the activation of TNF-primed neutrophils, we studied the influence of specific mAb on TNF-α induced H_2O_2 release by neutrophils placed on HUVEC and BCS

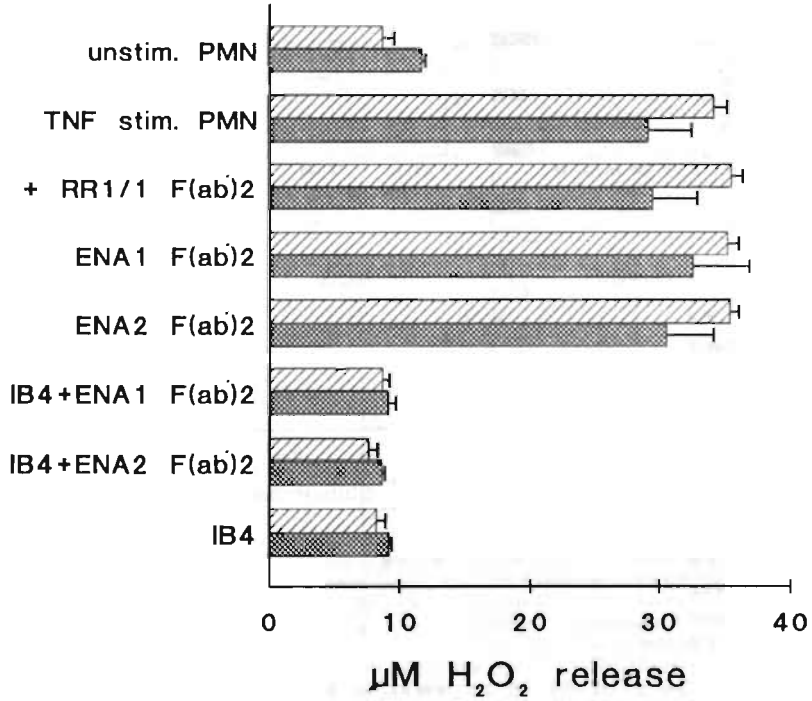


Figure 5. *Influence of anti ELAM-1 mAb (ENA1, ENA2), anti ICAM-1 mAb (RR1/1), and anti CD18 mAb (IB4) on H_2O_2 release by TNF-α stimulated neutrophils. Neutrophils were pretreated for 30min with 10ng/ml TNF-α at 4°C (TNF stim. PMN), or handled identically without TNF-α addition (unstim. PMN), and added to unstimulated HUVEC (hatched bars) or HUVEC activated with 10ng/ml TNF-α for 4h (cross hatched bars). Expression of ELAM-1 and ICAM-1 was confirmed by ELISA in parallel microtiter plates. Monolayers were incubated for 5min at RT with $F(ab')_2$ fragments of the anti ELAM-1 mAb or anti ICAM-1 mAb, directly before adding neutrophils with or without the intact anti CD18 mAb. H_2O_2 release was measured after 3h at 37°C. Data indicate mean and SD of 4 replicates. A representative experiment out of a series of 4 is shown.*

coated plastic. TNF-α pretreatment augmented neutrophil H_2O_2 release on endothelial cell monolayers *(Fig. 4 insets A and B)*, and on BCS coated plastic (data not shown) in a clear time and concentration dependent manner . Intact as well as $F(ab')_2$ fragments of mAb directed against CD18 and of mAb 904 directed against the LPS binding region of CD11b, were found to prevent the TNF-α induced increase in H_2O_2 release on HUVEC *(Fig. 4 main figure and inset C)*.

Again, presence of mAb directed against CD11a, CD11c and presence of the other two anti CD11b mAb during the assay did not decrease H_2O_2 release. mAb inhibition-levels, using either BCS coated plastic or HUVEC grown on fibronectin coated plastic as a substrate for neutrophil adherence, were mainly identical, indicating that CD11b/CD18 mediated contact-activation of TNF-primed neutrophils did not engage a ligand restricted to HUVEC.

Furthermore, we determined whether TNF-α stimulated neutrophils released H_2O_2 in presence of anti CD18 mAb, after increasing CD18 independent adhesion by addition of PHA or by 4h TNF activation of HUVEC. Neither presence of PHA nor 4h TNF activation of HUVEC induced a clear increase in neutrophil H_2O_2 release.

Also, the increase in H_2O_2 release induced by TNF-α pretreatment could, in both situations, still be reduced to basal levels by anti CD18 mAb *(Fig. 2C and 5)*. Blocking interaction between ELAM-1 or ICAM-1 and neutrophils using specific mAb did not affect the release of H_2O_2 by TNF-activated neutrophils *(Fig. 5)*, demonstrating the absence of engagement of ELAM-1 and ICAM-1 in H_2O_2-release by TNF-activated neutrophils. Identical H_2O_2-release levels were obtained using neutrophils which were activated by coincubation with TNF during the assay. No influence in neutrophil H_2O_2-release was found by placing neutrophils on 4h TNF activation of HUVEC, or by subsequent addition of anti ELAM-1 and of anti ICAM-1 antibody-fragments (data not shown).

Discussion

The cytokine TNF-α is reported to be a powerful stimulus for neutrophil mediated endothelial cell damage (7) and neutrophil H_2O_2 release (10, 11, 14, 15). Both endothelial cell damage and neutrophil H_2O_2 release require neutrophil-substratum interaction (10, 11, 14, 15, 34), a process which might involve known adhesion mediating structures, like the CD11/CD18 complex and the inducible endothelial cell adhesion molecules ELAM-1 and ICAM-1.

The role of these molecules in TNF-α induced neutrophil mediated endothelial cell detachment and H_2O_2 release, as facets of neutrophil toxicity, was investigated here by assessing the influence of mAb on the adhesion to endothelium, on endothelial cell detachment by neutrophils and on H_2O_2 release by neutrophils.

Two mAb reactive with CD11a were tested and found not to interfere with TNF-induced neutrophil activation, although they reduced adherence of TNF-stimulated neutrophils to HUVEC. Furthermore, mAb RR1/1 reactive with the ligand for CD11a on endothelial cells, ICAM-1, did not decrease neutrophil mediated endothelial cell detachment or neutrophil H_2O_2-release. The CD11a/CD18 or LFA-1 dimer therefore appeared not to be involved in activation of TNF-primed neutrophils. This finding is in concordance with previous literature on zymosan and FMLP induced neutrophils activation, in which anti CD11a mAb did not reduce activation (38-40).

For neutrophils, the function of CD11a/CD18 dimer and of ICAM-1 therefore seems to be limited to adherence and monolayer-transmigration (21), although CD11a/CD18 is capable of triggering cellular activation of lymphocytes (27, 41) and monocytes (42).

Up to 95% inhibition of endothelial cell detachment induced by TNF-activated neutrophils, and of H_2O_2-release by TNF-α activated neutrophils on either endothelium or BCS coated plastic was achieved by several mAb directed against the common β-chain of the CD11/CD18 hetero-dimer. Intact antibodies or $F(ab')_2$ fragments of mAb 904,

directed against the LPS binding region of CD11b (32, 37), reduced these neutrophil activities for 40 to 75%. The CD11b/CD18 integrin, also known as complement receptor 3 or Mac-1, therefore appeared to have a major role in mediating endothelial cell detachment and H_2O_2-release by TNF-activated neutrophils. Involvement of CD18 in adherence dependent H_2O_2 release by TNF-activated neutrophils has been worked out by Nathan et al. (14, 43), and confirms our observations. In contrast with our results, Nathan et al. found no influence of addition of intact mAb 904 on H_2O_2-release by TNF-activated neutrophils (14). Although we have no explanation for this discrepancy, CD11b involvement in TNF-induced neutrophil activation appears to be presumable, since CD11b/ CD18 was found to be crucial in activation of neutrophil H_2O_2-release by other stimuli such as FMLP (38) and zymosan (39, 40), and activation via CD18 is generally considered to depend on the ligand-interaction via one of its α-chains.

CD11b has two binding sites, each engaged in the binding of distinct classes of ligands (32, 37). One site is capable of recognizing Arg-Gly-Asp containing proteins, such as C3bi, fibrinogen, Leishmania gp 63 and an unidentified ligand on endothelial cells (44). Interactions between these ligands and CD11b do not induce neutrophil respiratory burst activity (40, 45). The other domain recognizes bacterial LPS, lipid A, zymosan and protein coated plastic, and is engaged in chemotaxis and respiratory burst activation (32, 37, 40).
We observed an inhibition of TNF-induced neutrophil toxicity by mAb 904, reactive with the latter domain, whilst two other anti CD11b mAb remained uneffective. Specific involvement of the LPS-binding region in a crucial, adherence induced, activation signal, passed to the neutrophil via CD18, thus appears to be the most logical explanation for this finding. The observed absence of inhibition of neutrophil mediated activation together with significant reductions in adherence by the anti CD11b mAb 44 and PDV11.2 might depend on an inhibition of ligand-interactions with the Arg-Gly-Asp binding region in absence of inhibition of the function of the LPS-binding region of CD11b. Further evidence that signal transduction instead of passive adherence is the specific function of CD11b/CD18 in TNF-induced neutrophil activation, was obtained by showing that CD18 independent adherence via addition of PHA, or by induction of ELAM-1 expression, could not overcome the inhibition of TNF-α induced neutrophil toxicity by anti CD18 mAb.

One to five hour TNF-α or IL-1 preincubation of endothelium has been described to induce an increase in neutrophil mediated endothelial injury (46, 47), whilst others found no influence on endothelial cell injury and neutrophil activation (6, 48). We therefore investigated whether 4 hour TNF-preincubation of endothelium, and more specifically, whether *de novo* ELAM-1 expression, and increased ICAM-1 expression by 4 hour TNF-preincubated endothelium could trigger neutrophil mediated endothelial cell detachment and neutrophil H_2O_2-release.
We report here that neutrophil interaction with neither ELAM-1 nor ICAM-1 contributes to neutrophil toxicity, since mAb

against these molecules did not reduce endothelial cell injury or H_2O_2-release by either unstimulated or TNF-preincubated neutrophils. Furthermore we showed that 4 hour TNF-preincubation of HUVEC did not increase H_2O_2-release by neutrophils placed un such endothelium, and thus extended our previous observation that TNF-activation of endothelial cells did not increase there vulnerability to, or trigger additional injury by TNF-α stimulated neutrophils (7).

The importance of the CD11/CD18 integrin for tissue injury in vivo has been demonstrated in a number of animal models. Addition of anti CD18 mAb showed to reduce tissue injury as well as mortality in ischaemia reperfusion induced shock in rabbits and monkeys (49, 50), and in bacterial meningitis in rabbits (51). In one study, the anti CD11b mAb OKM1 was shown to reduce the myocardial infarct-size in dogs (52).
Specific engagement of CD11b/CD18, via a pathway in which interactions with the lectin-like site of CD11b result in an CD18 transduced activation of previously primed neutrophils might thus be a major mechanism responsible for ischaemia or endotoxin induced tissue injury. In contrast, induction of local entrapment and transendothelial migration of leukocytes, without triggering local injury, might be the main function of increased ELAM-1 and ICAM-1 expression by activated endothelial cells.
A more detailed understanding of the function of the CD11/CD18 heterodimer en of endothelial cell adhesion molecules could add to the therapeutic potential for the management of syndromes with a neutrophil injury involving pathogenesis, like multiple organ failure, or acute allograft rejection.

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Chapter 5.4

ENDOTHELIAL CELL ASSOCIATED PLATELET-ACTIVATING FACTOR (PAF), A CO-STIMULATORY INTERMEDIATE IN TUMOR NECROSIS FACTOR-α (TNF) INDUCED H_2O_2 RELEASE BY ADHERENT NEUTROPHIL LEUKOCYTES

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Summary

TNF is a strong secretagogue for surface-contacting neutrophils. During inflammation, endothelium offers the first substrate for neutrophil adherence and for modulation of the toxic response of neutrophils to soluble agonists such as TNF. In this *in vitro* study, evidence is presented that endothelium participates actively in TNF-induced neutrophil respiratory burst activity, by expressing PAF in response to initial neutrophil H_2O_2 release. Three findings are shown, which favor such a mechanism. Firstly, PAF receptor antagonists reduced H_2O_2 release by TNF-activated neutrophils placed on endothelium for about 50%, whilst H_2O_2responses by neutrophils placed on serum-coated polystyrene remained intact. Secondly, preincubation of HUVEC with known PAF-inducing agents PMA, H_2O_2 and thrombin, followed by fixation,enhanced neutrophil H_2O_2 release in response to TNF. H_2O_2-releaseby these neutrophils was sensitive for presence of PAF-receptor antagonists, whilst H_2O_2-release from neutrophils placed on fixed non-activated endothelial cells was not. Finally, replacing endothelium by monolayers of human renal cortical epithelial cells and human fibroblasts, cells which are known to produce less PAF than endothelial cells, reduced the effect of PAF receptor antagonists. P-selectin expression and IL-8 release, two other ways by which endothelial cells might interfere with H_2O_2-release by TNF-preincubated neutrophils, were examined in parallel, and were found not to influence TNF-induced neutrophil H_2O_2-release.

We conclude that during neutrophil-endothelial interaction in inflammation, endothelium modulates the toxic response of neutrophils to TNF. Endothelial cell associated PAF, but not endothelial cell IL-8 release and P-selectin expression, is likely to participate in TNF-induced neutrophil respiratory burst activity.

Introduction

TNF-induced neutrophil respiratory burst activity is associated with highly flattened substrate-adherent neutrophils (1-4). It is not understood how this highly-adhesive status of neutrophils cooperates with signals from TNF-receptors in triggering respiratory burst activity. The following steps all appear essential in facilitating TNF induced neutrophil respiratory burst activity: a sustained fall in cAMP (2), reorganization of the cytoskeletal protein actin into focal subcortical F-actin at adhesive sites (2), and specific CD11b/CD18 mediated substrate interaction (1, 5).

This specific substrate interaction by the β_2 integrin member CD11b/CD18 requires a certain substrate composition (not just ligand-presence) (1, 6) and requires shedding of the neutrophil sialoprotein CD43, which, in its normal expression, antagonizes neutrophil spreading (3).

TNF-induced neutrophil respiratory burst activity has been mainly investigated using protein coated polystyrene as an *in vitro* model. CD11b/CD18 has a binding site which can directly bind polystyrene (7), and neutrophil interaction with polystyrene could alter respiratory burst responses.

Endothelium has anti-adhesive properties in comparison to protein coated plastic (8), and endothelium does not offer a ligand for the binding site of CD11b/CD18 which has affinity for plastic and microbial products (7). Whether neutrophils in contact with more physiological surfaces, such as a monolayer of endothelial cells, respond differently to TNF, is unknown. Previously we showed that HUVEC-adherent neutrophils show a CD11b/CD18 mediated H_2O_2-response to TNF which is similar to the response of neutrophils placed on coated polystyrene (5).

In this study the contribution of HUVEC tothis response is analyzed. Evidence is shown suggesting that HUVEC have a co-stimulatory role in TNF-induced neutrophil H_2O_2-release, by expressing membrane associated PAF.

Materials and Methods

Reagents and antibodies

rhTNF-α was kindly provided by BASF/Knoll Ag. (Ludwigshafen, FRG). PMA was purchased from Sigma (St. Louis, MO). Thrombin was kind gift of Dr. V. van Hinsbergen (Gabius Institute, Leiden, The Netherlands). rhIL-8 was a kind gift of Dr. J. Lindley (Sandoz Forschungsinstitut, Wien, Austria). WEB2086 was a kind gift of Dr. H. Heuer (Boeringer Mannheim GmbH, Mannheim, FRG). CV6209 and CV3988 were gifts from Takeda Chemical Industries (Osaka, Japan). Stock solutions of PAF receptor antagonists were prepared fresh for each experiment, by dissolving these agents in 0.9% NaCl at 10^{-4}M at RT (suspensions of CV3988 was heated to 50°C for 30 min todissolve). The following antibodies were employed: Polyclonal goat anti IL-8, a gift of Dr. J. van Damme (Rega Institute, University of Leuven, Leuven, Belgium); mAb CLB-C2, IgG_1, reactive with P-selectin (9), a gift of Dr. D. Roos (CLB, Amsterdam, The Netherlands); mAb G1, IgG_1, reactive with P-selectin (10), a giftof Dr. R. P. McEver (University of Oklahoma, Oklahoma, USA); mAb IB4 (IgG_{2a}), reactive with CD18 (11), a gift of Dr. M. Daha (University Hospital Leiden, The Netherlands); mAb 904 (IgG_1)

reactive with CD11b (12), a gift of Dr. J.D. Griffin (Dana Farber Cancer Institute, Boston, MA). $F(ab')_2$ fragments were prepared by pepsin digestion (Immobilized Pepsin, Pierce, Rockford, IL).

Cells

HUVEC were obtained by collagenase treatment of the human umbilical vein. The cells were cultured in fibronectin-coated (fibronectin kindly provided by Dr. J. van Mourik, CLB, Amsterdam, The Netherlands) tissue culture flasks (Costar, Cambridge, MA) inRPMI1640 (Gibco Europe, Paisley, Scotland), supplemented with 10% heat-inactivated human serum, 10% heat-inactivated bovine calf serum (BCS) (Hyclone, Logan, UT), 50µg/ml heparin (Sigma), 30µg/ml endothelial growth supplement (Collaborative Research Incorporated, Bedford, MA) and antibiotics. Endothelial cells were characterized by their pavement-like monolayer morphology and by positive staining with an anti E-selectin mAb after 4 h incubation with TNF. Cells of passage 3 to 4 were used for the experiments.

Human renal cortical epithelial cell explant cultures were obtained using previously described techniques (13). Briefly, small biopsies from the human renal cortex were obtained from kidneys not suitable for transplantation for technical reasons. The capsula was removed and the cortex-tissue was cut into small pieces, which were incubated in 0.25% trypsin for 25 minutes at 37°C.
The mixture was then filtered through one layer of sterilegauze, the filtrate was centrifuged, and the pellet was resuspended in RPMI-1640 supplemented with 10% heat-inactivated BCS and antibiotics. The mixture was placed in fibronectin coated tissue culture flasks. After culturing, a cell population characterized by a pavement-like monolayer morphology and by positive staining with anti-cytokeratin mAb was obtained. Endothelial cell contamination was absent as concluded from negative staining with an anti E-selectin mAb after 4 h incubation with TNF. Cells of passage 3 to 4 were used for the experiments.

Human fibroblasts (kindly provided by the department of Human Genetics, University of Limburg, The Netherlands) were propagated in RPMI-1640 supplemented with 10% BCS and antibiotics in fibronectin coated tissue culture flasks and characterized bytheir spindle-like morphology. Endothelial cell contamination was absent, as concluded from negative staining with an anti E-selectin mAb after 4 h incubation with TNF.
Cells of passage 6 to 10 were used for the experiments.

Neutrophils were prepared from buffy coats of donor blood kindly provided by the local blood bank, using standard techniques of buoyant density centrifugation on Lymphoprep (Nycomed, Oslo, Norway). Neutrophils were separated from erythrocytes by density gradient centrifugation on percoll (1.08g/ml, Pharmacia, Uppsala, Sweden). Preparations of neutrophils contained <5% contaminating cells, as evaluated after May Grunwald Giemsa staining.

H_2O_2 measurement

A previously described colorimetric method to determine H_2O_2 production was used (5). In short, neutrophils, reagents and mAb were suspended/dissolved in RPMI-1640 supple-

mented with 10% BCS, antibiotics, 200µg/ml phenol red (Sigma) and 8.5u/ml Horse radish peroxidase (type VI, Sigma). Neutrophils and reagents were added to microtiter-plates coated with BCS at 2.5 x 10^5 cells/well to a final volume of 200µl/well, and incubated for 3h at 37°C. 50µl 2MNaOH was added and OD was determined at 620nm. The lower sensitivity of the assay was 1.6µM H_2O_2.

Results

PAF-receptor antagonists reduce H_2O_2 release by TNF stimulated neutrophils in contact with HUVEC

As demonstrated previously (5, 14), neutrophil preincubation with 1 to 10ng/ml TNF-α resulted, after a lag phase of about one hour, in massive H_2O_2-release by neutrophils placed on a HUVECmonolayer. To investigate whether PAF is involved in this process, three PAF-receptor antagonists were employed in concentrations which interfere with exogenous PAF induced neutrophil activation (15-17). Neutrophils were pre-incubated with 10ng/ml TNF-α for 30 minutes at 4°C in polypropylene-tubes, washed, and placed inmicrotiter-plates on either BCS-coated plastic or on a confluent HUVEC-monolayer for 3 hours at 37°C in the presence or absence of different concentrations of PAF-receptor antagonists.

When neutrophils were placed on endothelium, 10^{-6}M of PAF receptorantagonist CV6209 reduced TNF induced H_2O_2-release from about 17µM to 5µM *(Fig. 1)*.

Addition of similar amounts of PAF receptor antagonist WEB2086 reduced H_2O_2-release from 18µM to 9µM *(Fig. 1)*.10^{-6}M of PAF receptor antagonist CV3988 also induced up

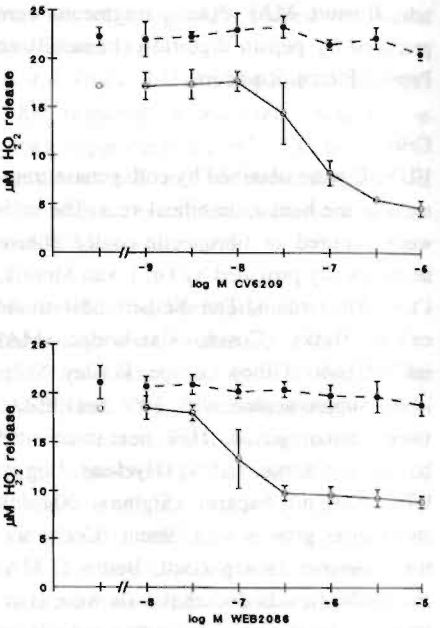

Figure 1: *PAF receptor antagonist CV6209 and WEB2086 inhibit H_2O_2-production by 10ng/ml TNF pre-incubated neutrophils placed on HUVEC coated wells (O) but not on BCS coated wells (●). H_2O_2-concentrations were measured after 3 hours of incubation.*
Data indicate mean and SD of quadruplicate measurements. If SD bars are not shown, they fall within the symbol.

to 50%inhibition of TNF-induced neutrophil H_2O_2-release, whilst similar amounts of WEB1090, an analog of WEB2086 without affinity for the PAF-receptor, did not influence TNF-induced neutrophil H_2O_2-release (data not shown).

The effect of PAF-receptor antagonists was relatively stronger after sub-maximal stimulation of neutrophil H_2O_2-release; addition of CV6209 nearly completelyinhibited neutrophil H_2O_2-release after preincubation of

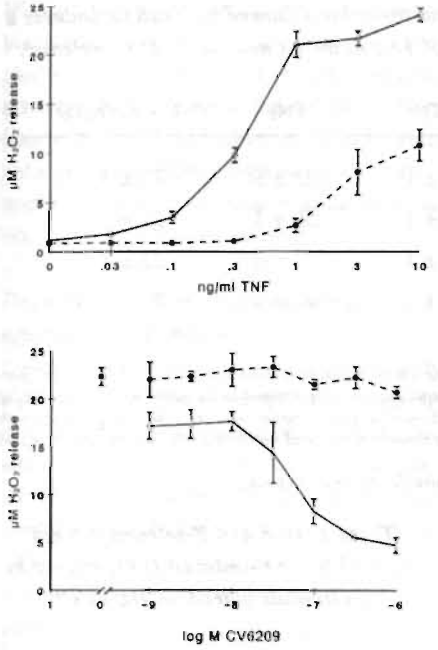

Figure 2: PAF receptor antagonist CV6209 reduces H_2O_2-production by neutrophils activated with different concentrations of TNF. Neutrophils were preincubated in polypropylene-tubes with the indicated concentrations of TNF for 30 minutes at 4°C, washed, and added alone (O) or in combination with 10^{-6}M CV6209 (●) to HUVEC-coated wells. H_2O_2-concentrations were measured after 3 hours of incubation.
Data indicate mean and SD of quadruplicate measurements. If SD bars are not shown, they fall within the symbol.

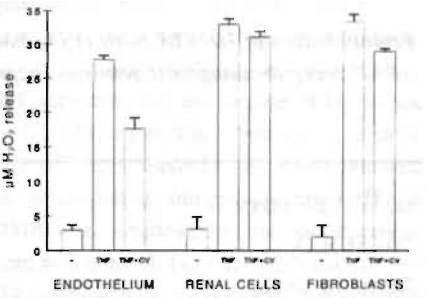

Figure 3: Comparison of the inhibitory effects of CV6209 on H_2O_2-production by neutrophils placed on monolayers of HUVEC, renal epithelial cells and fibroblasts. Neutrophils were preincubated with TNF and H_2O_2-concentrations were measured after 3 hours of incubation with or without 10^{-6}M CV6209. Data indicate mean and SD of quadruplicate measurements.
** $P<0.01$, when compared to H_2O_2-release in absence of CV6209, as determined by the unpaired one-tailed Student's t-test.*

neutrophils with 30 to 300pg/ml TNF *(Fig. 2)*. When BCS-coated plastic was used instead of endothelium as a substrate for neutrophil adherence, no inhibitory effect of CV6209, WEB 2086 *(Fig. 1)* and CV3988 (data not shown) was found, suggesting involvement of PAF in endothelial cell-neutrophil rather than in neutrophil-neutrophil interaction.

Comparison of different cell-types as a substrate for neutrophil-adherence

Endothelial cells are potent PAF-producers, in comparison to other non hemopoietic cells (18). To gain further evidence for a specific role of endothelial cells in PAF-receptor antagonist sensitive TNF-induced neutrophil H_2O_2-release, we comparedendothelium with monolayers of human renal epithelial cells and human fibroblasts. TNF preincubated neutrophils were added to monolayers of these three cell-types, and H_2O_2-release wasmeasured in presence and absence of 10^{-6}M CV6209.

Significant reductions in the amount of H_2O_2 produced after 3 hours of incubation at 37°C were found selectively when HUVEC were used asa substrate for neutrophil adherence *(Fig. 3)*, although on both renal cells and fibroblasts, addition of CV6209 resulted consequently in slightly reduced H_2O_2 amounts as experiments were repeated.

Table 1:

Preincubation of HUVEC with H_2O_2, PMA and thrombin,followed by fixation, induces a PAF receptor antagonist-sensitive increase of TNF-induced neutrophil H_2O_2-release[a].

	no TNF	TNF	TNF + CV6209
no HUVEC preinc.	1 ± 1[b]	23 ± 3	23 ± 3
20mM H_2O_2	2 ± 1	50 ± 1	21 ± 2
2U/ml thrombin	1 ± 1	42 ± 2	22 ± 3
10ng/ml PMA	1 ± 1	37 ± 2	19 ± 2

[a]H_2O_2, thrombin, PMA and control medium (no HUVEC preinc.) were added in a small volume to HUVEC coated, pre-warmed wells containing 200µl culture-medium, to the final concentrations mentioned in the table. After 30 minutes of incubation at 37°C,the 96-well microtiter-plates were placed on melting ice, rinsed with ice-cold PBS enriched with Ca^{++} and Mg^{++}, incubated with 0.1%glutaraldehyde for 10 minutes, rinsed, and incubated with neutrophils, 0.5ng/ml TNF, and 10^{-6}M CV6209 for 3 hours at 37°C.

[b]Data are expressed in µM and indicate mean and SD ofquadruplicate measurements.

HUVEC-activation with PMA, H_2O_2 and thrombin, followed by fixation induces a PAF-receptor antagonist sensitive increase in TNF-induced neutrophil H_2O_2-release

Since PMA, H_2O_2 and thrombin are known inducers of endothelialcell PAF-production (19-21), and membrane-associated PAF remains functionally intact after fixation (22), we tested whether preincubation of HUVEC with these agents, followed by rapid cooling of the monolayers and fixation with 0.1% glutaraldehyde, would prime neutrophil H_2O_2 release in response to TNF. On fixed-unstimulated HUVEC TNF-induced neutrophil H_2O_2-release was low, and no influence of CV6209 was measured.

Preincubation of HUVEC for 30 minutes at 37°C with either 20mM H_2O_2, 2U/ml thrombin, or 10ng/ml PMA enhanced neutrophil H_2O_2-release in response to 1ng/mlTNF, whilst preincubation was not sufficient to trigger H_2O_2-release by unstimulated neutrophils (Table 1). H_2O_2-release by TNF-

Table 2: IL-8 and P-selectin are not engaged in TNF-induced H_2O_2-release by neutrophils placed on HUVEC[a].

	no TNF	TNF
No agent	3 ± 1[b]	52 ± 1
100ng/ml IL-8	3 ± 1	50 ± 2
anti IL-8 ab	3 ± 1	53 ± 1
anti P-selectin mAb C2	3 ± 1	54 ± 1
anti P-selectin mAb G1	3 ± 1	54 ± 1
anti P-selectin F(ab')$_2$ G1	3 ± 1	54 ± 1
anti CD18 F(ab')$_2$ IB4	4 ± 1	5 ± 1
anti CD11b F(ab')$_2$ 904	3 ± 1	39 ± 7
10^{-6}M WEB 2086	4 ± 1	37 ± 5

[a]HUVEC were incubated with antibodies (final concentration 5µg/ml) and WEB 2086 for 5 minutes at RT, and neutrophils and TNF (final concentration 0.5ng/ml) were added as indicated in the table. H_2O_2-concentrations were measured after 3 hours of incubation at 37°C and are expressed in µM.

[b]Data indicate mean and SD of quadruplicate measurements.

stimulated neutrophils placed on fixed HUVEC activated with either H_2O_2, thrombin or PMA, was again sensitive for CV6209 *(Table 1)*. In presence of 10^{-6}M CV6209, priming effects ofendothelial cell preincubation with H_2O_2, thrombin and PMA were nolonger detectable, suggesting that neutrophil priming by activated endothelial cells was completely PAF-mediated.

P-selectin and IL-8 do not influence neutrophil H_2O_2-release

Endothelial cells respond to stimulation with H_2O_2, PMA andthrombin with rapid and *de novo* membrane expression of the neutrophil adhesion molecule P-selectin, which parallels PAF expression (20, 23-26). Another candidate for endothelial cell interference with neutrophil activation is IL-8, which is released in high amounts by endothelial cells activated with TNF, IL-1 and microbial products (27-29), and is thought to mediate neutrophil emigration from the vasculature by forming a chemotactic gradient from the luminal side to the basal side of the endothelium (30, 31). To investigate whether P-selectin and IL-8 might influence H_2O_2 release by neutrophils in contact with HUVEC, we investigated the influence of rhIL-8, of goat polyclonal antibodies which inhibit the biological activity of IL-8, and of two monoclonal antibodies reactive with P-selectin in our model. Addition of100ng/ml rhIL-8 neither induced neutrophil H_2O_2-release, nor enhanced TNF induced neutrophil H_2O_2-release *(Table 2)*. In agreement with the absence of a role for IL-8 on TNF-induced neutrophil H_2O_2-release, we found that IL-8 reactive antibodies were unable to reduce H_2O_2 release by HUVEC-contacting neutrophilsin presence of TNF *(Table 2)*.

Addition of mAb C2, mAb G1 and $F(ab')_2$ fragments of mAb G1, in concentrations which inhibited P-selectin-mediated neutrophil adhesion, did not reduce H_2O_2-release by HUVEC-contacting neutrophils in presence of TNF *(Table 2)*. mAb reactive with the neutrophil $ß_2$ integrin member CD11b/CD18 were employed in the same experiment as a control. $F(ab')_2$ fragments of mAb IB4 reactive with CD18 nearly completely reduced neutrophil H_2O_2-release, whilst $F(ab')_2$ fragments of mAb 904 partially reduced neutrophil respiratory burst activity *(Table 2)*, which is in line with previous observations (5).

Addition of isotype-matched control antibodies did not influence H_2O_2-release (data not shown).

Discussion

In this study, we demonstrated that endothelial cells can regulate TNF-induced respiratory burst activity by contacting neutrophils, by a PAF-receptor antagonist sensitive mechanism. In our *in vitro model* at least 50% of the H_2O_2 production by TNF-activated neutrophils was dependent on endothelial cell activity. This endothelial cell contribution could by blocked by preventing activation of endothelial cells by fixation of the monolayer.

It was restored if, before fixation, endothelial cells were incubated with PAF-inducing agents, and blocked again by subsequent addition of PAF-receptor antagonists, which suggests that endothelial cell associated PAF mediates the increase in TNF-induced neutrophil H_2O_2-release. In line with these results, we showed thatneutrophil H_2O_2-release was not PAF-receptor antagonist

sensitive in absence of bystander-cells, and probably moderately PAF-receptor antagonist sensitive using fibroblasts or RCEC as bystander cells.

Endothelial cells are well known producers of PAF. In response to thrombin, reactive oxygen species, histamine, leukotrienes and PMA, endothelial cells produce PAF within minutes (19-21). TNF, IL-1 and LPS also trigger endothelial cell PAF production, although to a lesser extend and at later time-points than the first group of agents (31-35). Endothelial PAF remains mainly cell-associated, and can be recognized by contacting neutrophils (36). H_2O_2-concentrations between 100µM and 10mM,which can be reached easily in the micro-environment of the contact-area between H_2O_2-producing neutrophils and endothelium, incite expression of membrane PAF within 10 to 30 minutes (19, 20).
Initial respiratory burst activity by TNF-activatedneutrophils thus might initiate an auto-amplification cycle, which is regulated by neighboring endothelial cells by expressing surface-associated PAF. Experiments using selective inhibitors ofthe NADPH-oxidase pathway are currently being conducted at our lab, to investigate whether respiratory burst products are indeed responsible endothelial cell PAF production.

Endothelial cell associated PAF is known to participate in regulating neutrophil attachment to activated endothelium. In parallel to rapid PAF production, endothelial cells respond to thrombin, reactive oxygen species, histamine, leukotrienes and PMA by *de novo* expression of P-selectin (20, 23-26).
In parallel to late PAF production, endothelial cells respond to TNF, IL-1 and LPS by *de novo* expression of E-selectin (34, 37, 38). PAF cooperates with P-selectin (23) and with E-selectin (39) in mediating increased neutrophil endothelial cell adherence, by triggering an increase in affinity of CD11b/CD18. During neutrophil interaction with E-selectin expressing endothelium, the role of PAF is, however, less essential since CD11b/CD18 'activation' can also be triggered by E-selectin alone (39, 40), or by IL-8, a neutrophil chemotaxin produced by endothelium activated with E-selectin inducing agents, but not by endothelium activated with the P-selectin inducing agents thrombin, reactiveoxygen species, histamine and leukotrienes (27-29). Other influences of endothelial cell associated PAF on neutrophil function are less clear. Neutrophil diapedesis through the endothelium is triggered clearly by E-selectin expressing endothelium (41-44) but hardly by P-selectin expressing endothelium (42, 45, 46). Presence of PAF, without simultaneous presence of IL-8, might be insufficient in triggering neutrophil transmigration (30, 31).

Besides its role in neutrophil endothelial cell adherence and transmigration, endothelial cell associated PAF might influence neutrophil respiratory burst and protease release. Like neutrophil chemotaxins such as IL-8, C5a and FMLP, nM concentrations of PAF can be chemotactically active, can induce CD11b/CD18 activation and upregulation, and can prime neutrophils for increased responses to other chemotaxins (47-50).
Also like other chemotaxins, µM concentrations of PAF can trigger a short and low respiratory burst in non adherent neutrophils, which can be increased by previous incuba-

tion of neutrophils with cytochalacins (49-51). This in contrast to neutrophil secretagogues such as TNF, which at nM concentrations lack chemotactic activity (52-54), but induce a highly adhesive status, associated with prolonged massive oxygen radical release, which is inhibited in stead of increased by cytochalacin-presence (2, 55).

TNF-induced adhesion dependent neutrophil respiratory burst activity has, until now, not been described to be increased by presence of chemotaxins. Endothelial cell released or added IL-8 (this study), C5a (own unpublished observation), FMLP (52), P-selectin binding (this study) or E-selectin binding (5), known to function as a 'tethered chemotaxin' (40), all fail to increase TNF-induced adherence dependent neutrophil respiratory burst activity.

Moreover, previous activation by such agonists decreases the sensitivity of neutrophils for TNF, by inducing rapid shedding of the TNF-receptor (57, 58). Our finding, that endothelial cell associated PAF can increase prolonged adhesion dependent TNF induced neutrophil-H_2O_2 release thus defines a functional effect of PAF on neutrophils which distinguishes PAF from other neutrophil-chemotaxins.

So far, two physiological mechanisms have been suggested which regulate neutrophil respiratory burst activity after TNF-activation. The first involves human serum albumin, which can antagonize TNF-induced neutrophil respiratory burst activity by binding to surface CD43. Albumin binding to CD43 prevents shedding of this negatively charged sialophorin, and thus prevents extensive neutrophil spreading and the subsequent TNF-induced respiratory burst (3).

Additionally, the composition of the substrate to which the neutrophil attaches, determines the duration of the lag phase before TNF-induced oxygen radical production starts, and influences its size (1, 6). The mechanism and physiological relevance of the influence of substrate-proteins such as collagen, fibronectin and vitronectin remains unclear.

A thirth co-regulatory mechanism is suggested in this study. Neutrophil-contacting endothelial cells, and probably to a lesser extend also other cells, can enhance ongoing TNF-induced neutrophil respiratory burst activity by expressing membrane-associated PAF. TNF-induced neutrophil toxicity thus is highly dependent on the environmental context, and is closely connected to PAF-mediated toxicity.

Both TNF (59-61) and PAF (62-64) have been claimed to mediate tissue and organ damage in clinical syndromes such as ischemia reperfusion injury and ARDS.

The costimulatory effect of PAF on TNF-induced neutrophil toxicity demonstrated in this study might be one of the levels at which PAF and TNF cooperate in these syndromes.

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INTERMEZZO

**Uit "Gödel, Escher, Bach:
een eeuwige gouden band"**

**(oorspronkelijke titel "Gödel, Escher, Bach: an eternal golden braid")
van Douglas R. Hofstadter**

Achilles: Ik weet dat jullie dit niet zullen geloven, maar het antwoord op de vraag staat pal voor onze neus, verborgen in de tekening. Het is maar één woord - maar wat een belangrijk woord: 'MU'!

Kreeft: Ik weet dat jullie dit niet zullen geloven, maar het antwoord op de vraag staat pal voor onze neus, verborgen in de tekening. Het is maar één woord - maar wat een belangrijk woord: 'HOLISME'!

Achilles: Zeg, wacht eens even. Je ziet ze vliegen. Het is zo klaar als een klontje dat de boodschap van deze tekening 'MU' is, en niet 'HOLISME!

Kreeft: Neem me niet kwalijk, maar mijn ogen zijn uitzonderlijk goed. Wil je nog eens kijken en zeg me dan of de tekening zegt wat ik zei dat ze zegt!

Miereneter: Ik weet dat jullie dit niet zullen geloven, maar het antwoord op de vraag staat pal voor onze neus, verborgen in de tekening. Het is maar één woord - maar wat een belangrijk woord: 'REDUCTIONISME'!

Kreeft: Zeg, wacht eens even. Je ziet ze vliegen. Het is zo klaar als een klontje dat de boodschap van deze tekening 'HOLISME' is, en niet 'REDUCTIONISME!

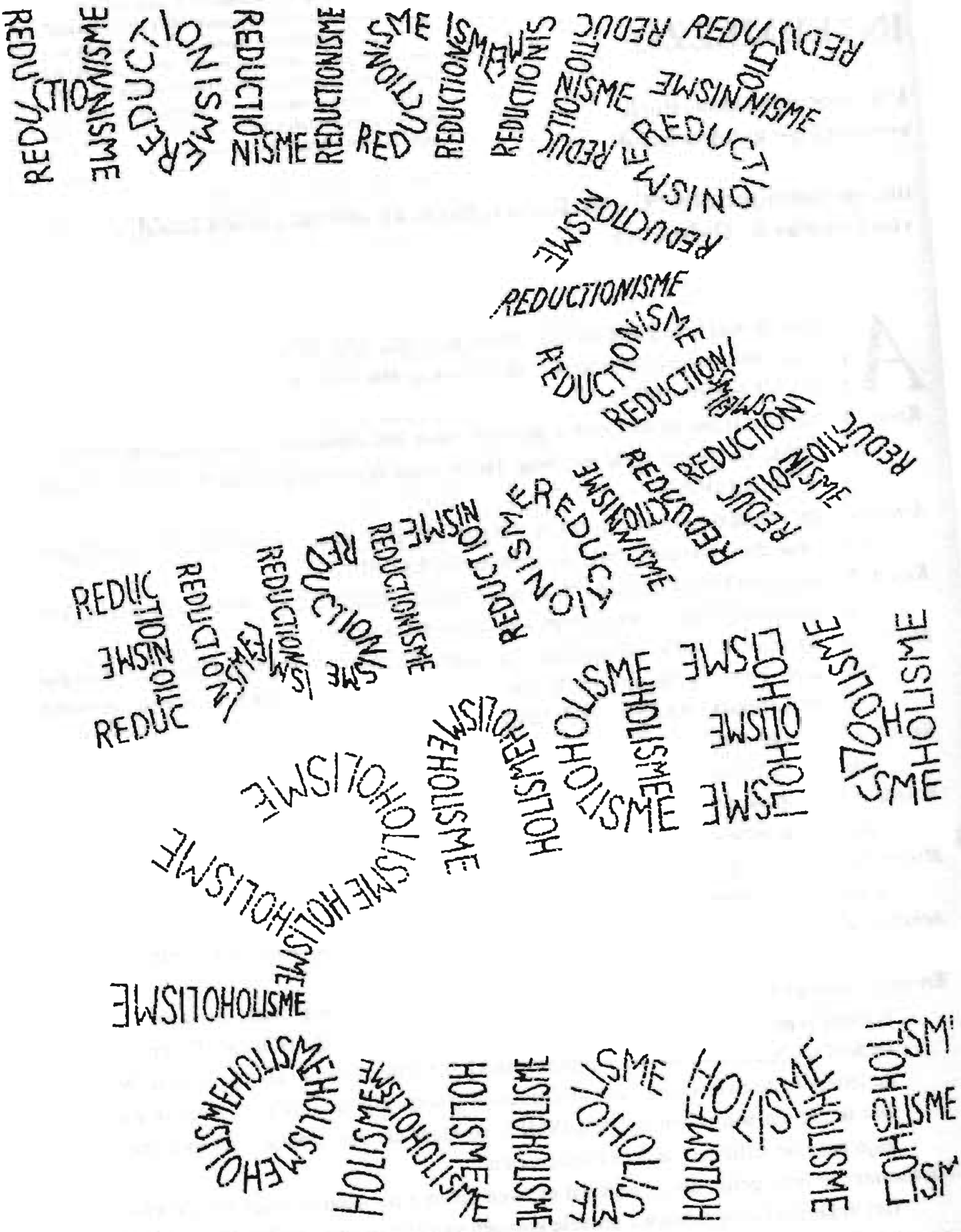
Achilles: Nog iemand die erin vliegt! niet 'HOLISME', niet 'REDUCTIONISME', maar 'MU' is de boodschap van deze tekening, dat staat vast.

Miereneter: Neem mij niet kwalijk, maar mijn ogen zijn uitzonderlijk scherp. Wil je nog eens kijken en zeg me dan of de tekening zegt wat ik zei dat ze zegt!

Achilles: Zie je dan niet dat de tekening is samengesteld uit twee delen, die beide een letter vormen?

Kreeft: Je hebt gelijk als je zegt dat er twee delen zijn, maar je interpretatie klopt niet. Het linkerdeel is geheel opgebouwd uit drie kopieën van één woord: 'HOLISME'; en het rechterdeel is opgebouwd uit vele kopieën, in kleine letters, van hetzelfde woord. Waarom de letters in de twee delen van verschillende grootte zijn weet ik niet, maar ik weet wel wat ik zie, en wat ik zie is 'HOLISME', zo klaar als een klontje. Het gaat me boven mijn pet hoe jullie iets anders kunnen zien.

Miereneter: Je hebt gelijk als je zegt dat er twee delen zijn, maar je interpretatie klopt niet. Het linkerdeel is opgebouwd uit vele kopieën van één woord: 'REDUCTIONISME'; en het rechterdeel opgebouwd uit één kopie, in grote letters, van hetzelfde woord. Waar-



[*Tekening Marjan Gerritse naar voorbeeld van de auteur.*]

om de letters in de twee delen van verschillende grootte zijn weet ik niet, maar ik weet wel wat ik zie, en wat ik zie is 'REDUCTIONISME', zo klaar als een klontje. Het gaat me boven mijn pet hoe jullie iets anders kunnen zien.

Achilles: Ik weet wat er hier aan de hand is. Jullie hebben alle twee letters gezien die andere letters vormen, of die zelf zijn gevormd uit andere letters. In het linkerdeel staan inderdaad drie 'HOLISME'S', maar die zijn elk weer gevormd uit kleinere kopieën van het woord 'REDUCTIONISME'. En op complementaire wijze zit er in het rechterdeel inderdaad een 'REDUCTIONISME', maar het is gevormd uit kleine kopieën van het woord 'HOLISME'. Dit is allemaal goed en wel, maar door jullie onnozele gekrakeel zagen jullie door de bomen het bos niet meer. Want wat heeft het voor zin om te kibbelen over 'HOLISME' of 'REDUCTIONISME' terwijl de enige manier om deze kwestie te begrijpen het trancenderen van de vraag is door te antwoorden: 'MU'?

Kreeft: Ik zie de tekening nu zoals jij hem hebt beschreven, Achilles, maar ik heb er geen flauw idee van wat je bedoelt met die vreemde uitdrukking: 'MU'.

Achilles: Ik wil jullie graag vertellen wat ik bedoel, als jullie mij eerst vertellen wat die vreemde uitdrukkingen 'HOLISME' en 'REDUCTIONISME' betekenen.

Kreeft: Het begrijpen van HOLISME is de natuurlijkste zaak van de wereld. Het is de opvatting dat 'het geheel groter is dan de som der delen'. Geen mens met een gezonde rechterhersenhelft kan holisme verwerpen.

Miereneter: Het begrijpen van REDUCTIONISME is de natuurlijkste zaak van de wereld. Het is de opvatting dat 'je een geheel volledig kan begrijpen als je de delen en de aard van hun "som" begrijpt'. Geen vrouw die niet twee linkerhersens heeft, kan reductionisme verwerpen.

Kreeft: Ik verwerp reductionisme. Ik daag je uit om mij te vertellen hoe je bij voorbeeld een stel hersenen reductionistisch moet verklaren. Iedere reductionistische verklaring van de hersenen zal onvermijdelijk te kort schieten als het gaat om de vraag waar het bewustzijn dat de hersenen ondervinden vandaan komt.

Miereneter: Ik verwerp holisme. Ik daag je uit om mij te vertellen hoe bijvoorbeeld een holistische beschrijving van een mierenkolonie meer verklaart dan een beschrijving van de mieren zelf, hun taken en hun onderlinge betrekkingen. Iedere holistische verklaring van een mierenkolonie zal onvermijdelijk tekort schieten als het gaat om de vraag waar het bewustzijn van een mierenkolonie vandaan komt.

Achilles: O nee! Nog een ruzie ontketenen was wel het laatste wat ik wilde. Maar nu ik de controverse begrijp, denk ik dat mijn verklaring van 'MU' uitkomst kan bieden. 'MU' is een oud Zen-antwoord dat, als antwoord op de vraag, de vraag ONVRAAGT. De vraag luidt hier volgens mij: 'Moet de wereld holistisch, of reductionistisch worden verklaard?' En het antwoord 'MU' verwerpt hier de premisse van de vragen, namelijk dat het een of het andere moet worden gekozen. Door de vraag te ontvragen openbaart zich een grotere waarheid: die van een ruimere context waarin zowel holistische als reductionistische verklaringen passen.

Miereneter: Absurd! Jouw 'MU' is even stom als het moe van een koe. Ik moet niks hebben

van die Zen-rimram.

Kreeft: Bespottelijk! Jouw 'MU' is zo onnozel als het mioe van een poes. Ik moet niets hebben van die Zen-ramrim.

Achilles: Lieve deugd! Zo komen we nergens. Waarom ben je zo eigenaardig stil, meneer Schildpad? Ik wordt er nogal zenuwachtig van. Jij bent toch zeker wel in staat om orde te scheppen in deze chaos?

Schildpad: Jullie zullen me niet geloven, maar het antwoord op de vraag staat pal voor onze neus, verborgen in de tekening. Het is maar één woord - maar wat een belangrijk woord: 'MU'!

Achilles: O meneer S., voor de eerste keer laat jij me in de steek. Ik dacht dat jij, die de dingen altijd zo goed ziet, in staat was dit dilemma op te lossen - maar kennelijk heb jij niet verder gekeken dan ik. Nou ja, ik denk dat ik al blij mag zijn dat ik voor een keertje even ver gekeken heb als meneer schildpad.

Schildpad: Neem me niet kwalijk, maar mijn ogen zijn opperbest. Wil je nog eens kijken en zeg me dan of de tekening zegt wat ik zei dat ze zegt!

Achilles: Natuurlijk wel! Je hebt alleen mijn eigen oorspronkelijke waarneming herhaald.

Schildpad: Misschien staat er 'MU' in deze tekening, maar op een dieper niveau dan jij je voorstelt, Achilles - een octaaf lager (figuurlijk gesproken). Maar op dit moment twijfel ik eraan of we onze onenigheid op zo'n abstract niveau kunnen bijleggen. Ik zou graag zien dat zowel het holistische als het reductionistische standpunt expliciet werden gemaakt; dan hebben we misschien een betere basis voor een beslissing. Ik zou bij voorbeeld bijzonder graag een reductionistische beschrijving van een mierenkolonie horen.

Kreeft: Misschien dat dr. Miereneter je iets wil vertellen over zijn ervaringen daarmee. Per slot van rekening is hij beroepshalve een soort expert op dat gebied.

Schildpad: Ik ben ervan overtuigd dat we veel van je kunnen leren, dr. Miereneter. Zou je ons meer kunnen vertellen over mierenkolonies uit reductionistisch perspectief.

Miereneter: Met alle genoegen. Zoals meneer Kreeft reeds vertelde, heb ik beroepsmatig de mierenkolonies diepgaand onderzocht.

Achilles: Dat kan ik me voorstellen! Het beroep miereneter mag wel haast synoniem met expert op het gebied van mierenkolonies worden genoemd.

Miereneter: Pardon. 'Miereneter' is niet mijn beroep; het is mijn soort. Ik ben koloniechirurg van beroep. Mijn specialisme is het corrigeren van nerveuze spanningen in de kolonie door middel van de chirurgische ingreep.

Achilles: O, ik snap het. Maar wat versta jij onder 'nerveuze spanningen' bij een mierenkolonie?

Miereneter: De meeste van mijn cliënten lijden aan een vorm van spraakstoornis. Je weet wel, een kolonie die in alledaagse situaties steeds naar woorden moet zoeken. Dat kan heel tragisch zijn. Ik probeer de situatie te verbeteren door, eh, het aangetaste deel van de kolonie te verwijderen. Deze operaties zijn soms heel ingrijpend en het vergt een jarenlange studie voor je ze kunt uitvoeren.

Achilles: Maar - om te kunnen lijden aan spraakstoornissen moet je toch eerst een spraakvermogen hebben?

Miereneter: Dat klopt.

Achilles: Aangezien mierenkolonies niet over dat vermogen beschikken, sta ik een beetje verstomd.

Kreeft: Jammer, Achilles, dat je er vorige week niet bij was toen dr. Miereneter en tante Myra Hoop bij mij op bezoek waren. Had ik jullie toen maar uitgenodigd.

Achilles: Is tante Myra Hoop een echte tante van je, meneer Kreeft?

Kreeft: O nee, ze is eigenlijk niemands tante.

Miereneter: Maar de lieve schat staat erop dat iedereen haar zo noemt, ook vreemden. Het is een van haar vele vertederende trekjes.

Kreeft: Ja, tante Myra Hoop is heel excentriek, maar het is zo'n aardige troel. Toch jammer dat jullie er vorige week niet bij waren.

Miereneter: Ze is beslist een van de meest ontwikkelde mierenkolonies die ik ooit heb leren kennen. Wij hebben samen vele avonden lang zitten praten over de meest uiteenlopende onderwerpen.

Achilles: Ik dacht dat miereneters mierenverslinders waren in plaats van beschermheren van het mierenintellect!

Miereneter: Ach, dat hoeft elkaar niet uit te sluiten. Ik sta op goede voet met mierenkolonies. Ik eet alleen MIEREN, geen kolonies - en dat komt beide partijen ten goede: mij en de kolonie.

Als ik, een miereneter, een bezoekje aan tante Myra Hoop kom brengen, raken al die dwaze mieren in paniek zodra ze mijn geur opsnuiven - en dat betekent dat ze totaal anders beginnen rond te rennen dan ze deden voor ik er was.

Achilles: Maar dat is begrijpelijk, want je bent een gevreesde vijand van de kolonie.

Miereneter: Ik moet toegeven dat iedere mier afzonderlijk bang voor me is; maar dat is een andere kwestie. Je ziet in ieder geval dat als reactie op mijn komst de interne verdeling van de mieren volkomen verandert.

Achilles: Dat is duidelijk.

Miereneter: En dat aanpassen aan de actuele situatie, weerspiegelt mijn aanwezigheid. Je kunt de verandering van oude toestand naar nieuwe beschrijven door te zeggen dat er een 'stuk kennis' aan de kolonie is toegevoegd.

Achilles: Hoe kun je de verdeling van verschillende types mieren over een kolonie nu een 'stuk kennis' noemen.

Miereneter: Daar raak je de kern van de zaak. Maar dat vergt enige nadere uitleg. Als je blijft denken in termen van de lagere niveaus - individuele mieren - zie je door de bomen het bos niet meer. Dat niveau is gewoon veel te microscopisch en als je microscopisch denkt, zal je onvermijdelijk sommige grootschalige kenmerken missen. Er zijn verschillende typen mieren, die 'kasten' genoemd worden, binnen iedere kolonie. Voor de beschrijving van de kasteverdeling zal je het juiste kader op hoog niveau moeten vin-

den. Alleen dan wordt duidelijk hoe de kasteverdeling vele stukken kennis kan coderen.

Achilles: Maar hoe vind je dan de juiste eenheden om de huidige toestand van de kolonie te beschrijven?

Miereneter: Goed, dan beginnen we van onder af aan. Als er bij mieren iets gedaan moet worden, vormen ze kleine 'ploegen', die samenwerken om een karwei te klaren. Zoals ik al eerder zei, vormen en ontbinden zich de hele tijd kleine groepen mieren. Groepen die een poosje bestaan zijn de ploegen, en ze vallen niet uiteen omdat er inderdaad iets voor ze te doen is. Als er ergens een onbeduidende hoeveelheid voedsel is, die wordt ontdekt door een rondtrekkende mier, die vervolgens haar enthousiasme probeert over te brengen op andere mieren, zal het aantal mieren dat reageert evenredig zijn met de hoeveelheid voedsel - en een onbeduidende hoeveelheid zal niet voldoende mieren aantrekken om de drempel te overschrijden.

Achilles: Ik snap het. Ik veronderstel dat deze 'ploegen' een van de structurele niveaus vormen die ergens tussen het niveau van de afzonderlijke mier en het niveau van de kolonie vallen.

Miereneter: Precies. Er bestaat een bijzondere ploeg die ik een 'signaal' noem - en alle hogere structurele niveaus berusten op signalen . In feite zijn alle hogere entiteiten verzamelingen van samenwerkende signalen. Er zijn ploegen op hogere niveaus waarvan de afzonderlijke leden geen mieren zijn, maar ploegen op lagere niveaus. Uiteindelijk kom je bij de ploegen op het laagste niveau - d.w.z. signalen - en daaronder vallen de mieren.

Achilles: Vanwaar die suggestieve naam 'signalen'?

Miereneter: Dat komt door hun functie. Signalen zorgen ervoor dat mieren met verschillende specialismen in de juiste afdeling van de kolonie terechtkomen. Het klassieke verhaal van een signaal luidt als volgt: het komt tot leven doordat de drempel die nodig is om te overleven wordt overschreden, dan verplaatst het zich over enige afstand door de kolonie en op een gegeven moment valt het min of meer uiteen in de individuele leden die aan hun lot worden overgelaten.

Achilles: Dat lijkt op een golf, die van verre zeesterren en wier meevoert en ze dan, hoog en droog, her en der op het strand achterlaat.

Miereneter: In zekere zin is dat hetzelfde, want de ploeg laat inderdaad iets achter dat hij over een bepaalde afstand met zich heeft meegevoerd, maar terwijl het water van de golf terugspoelt naar zee, is er in het geval van een signaal geen sprake van een dragende substantie, aangezien de mieren dat zelf zijn.

Schildpad: En ik veronderstel dat juist op de plek in de kolonie waar mieren van dat type nodig waren een signaal zijn samenhang verliest.

Miereneter: Natuurlijk.

Achilles: Natuurlijk? IK vind het niet zo natuurlijk dat een signaal altijd daarheen gaat waar er behoefte aan is. En ook al gaat het in de goede richting, hoe weet het dan waar het uiteen moet vallen? hoe weet het dat het is aangekomen?

Miereneter: Dat zijn uiterst belangrijke kwesties, want dan moet je een verklaring vinden voor het feit dat signalen doelmatig gedrag - of iets wat op doelmatig gedrag lijkt - ver-

tonen. Aan de hand van de beschrijving zou je geneigd zijn het gedrag van signalen te bestempelen als gedrag dat zich richt op het vervullen van een behoefte en het dan ook 'doelmatig' te noemen. Maar je kunt het ook op een andere manier bekijken.

Achilles: Wacht eens even. Het gedrag IS doelmatig, of het is het NIET. Het wil er bij mij niet in dat het beide kan zijn.

Miereneter: Laat me uitleggen hoe ik het zie, en daarna kunnen we kijken of we het eens zijn. Als een signaal eenmaal gevormd is, beseft het niet dat het in een bepaalde richting moet gaan. Maar hierbij is de verfijnde kasteverdeling cruciaal. Die bepaald juist de beweging van signalen door de kolonie en ook hoe lang een signaal stabiel blijft en waar het zal 'oplossen'.

Achilles: Alles berust dus op kasteverdeling.

Miereneter: Juist. Stel dat een signaal zich voortbeweegt. Terwijl het zich voortbeweegt ontstaat er een interactie tussen de mieren waar het uit bestaat, en de mieren van de lokale gebieden waar het doorheentrekt, hetzij door direct contact, hetzij door uitwisseling van geuren. Het contact en de geuren verschaffen informatie over urgente plaatselijke aangelegenheden, zoals het bouwen van een nest, broedzorg, of wat dan ook. Het signaal blijft hecht bijeen zolang de plaatselijke behoeften afwijken van wat het kan bieden; maar ALS het een bijdrage kan leveren, valt het uiteen en loost het ter plekke een frisse ploeg van beschikbare mieren. Zie je nu hoe de kasteverdeling fungeert als een overkoepelende instantie voor de ploegen binnen een kolonie?

Achilles: Ja, nu is het me duidelijk.

Miereneter: En zie je ook dat het bij deze interpretatie niet nodig is om doelmatigheid toe te schrijven aan het signaal?

Achilles: Ik geloof van wel. Ik begin de dingen nu ook van twee kanten te zien. In miere-ogen heeft een signaal GEEN doel. De typische signaalmier slentert een beetje door de kolonie, naar niets speciaals op zoek, totdat ze het gevoel krijgt dat ze moet stoppen. Haar ploegmaten zijn het daar gewoonlijk mee eens en op dat moment lost de ploeg zich op door uiteen te vallen, waarbij wel de leden overblijven maar de samenhang verdwijnt. Er is geen planning en voorbereiding nodig; er hoeft evenmin gezocht te worden naar de juiste richting. Maar voor de KOLONIE reageerde de ploeg louter op een boodschap, geschreven in de taal van de kasteverdeling. Zo gezien heeft het erg veel weg van doelmatige activiteit.

Kreeft: Wat zou er gebeuren als de kasteverdeling volkomen willekeurig was? Zouden signalen dan nog altijd binden en ontbinden?

Miereneter: Zeker wel. Maar de kolonie zou door de zinloosheid van de kasteverdeling geen lang leven beschoren zijn.

Kreeft: Dat is nou wat ik bedoelde. Kolonies houden stand omdat hun kasteverdeling zinvol is, en dat zinvolle is iets holistisch, dat op lager niveau niet zichtbaar is. Je verklaring verliest aan kracht als je geen rekening houdt met dat lagere niveau.

Miereneter: Ik begrijp wat je bedoelt, maar ik geloof dat je de dingen te beperkt ziet.

Kreeft: Hoezo?

Miereneter: Mierenkolonies zijn al miljarden jaren onderworpen aan de strenge wetten van de evolutie. Een paar mechanismen werden geselecteerd, maar de meeste werden weggeselecteerd. Het eindresultaat was een stel mechanismen die ervoor zorgen dat een mierenkolonie functioneert zoals we hebben beschreven. Als je het hele proces als een film zou kunnen zien - uiteraard een film die miljarden malen sneller loopt dan de werkelijkheid - zou je zien hoe het ontstaan van de diverse mechanismen een natuurlijke reactie is op druk van buitenaf, net zoals de belletjes in kokend water een natuurlijke reactie zijn op een warmtebron buiten. Ik denk niet dat je 'betekenis' en 'doel' ziet in de belletjes van kokend water - of wel soms?

Kreeft: Nee, maar -

Miereneter: Dat is nu wat IK bedoel. Hoe groot de luchtbel ook is , zij dankt haar bestaansrecht aan processen op moleculair niveau, en 'wetten op hoger niveau' kun je rustig uit je hoofd zetten. Hetzelfde geld voor mierenkolonies en hun ploegen. Als je de dingen beschouwt vanuit het weidse perspectief van de evolutie, ontneem je misschien de hele kolonie betekenis en doel. Dat worden dan overbodige begrippen.

Achilles: Maar dr. Miereneter, waarom vertel je me nu dan dat je met tante Myra Hoop sprak? Het lijkt nu wel of je ontkent dat ze zou kunnen spreken en denken.

Miereneter: Ik ben niet inconsequent, Achilles. Net als ieder ander heb ik er moeite mee om de dingen over een dergelijke immense tijdspanne te zien, en daarom vind ik het veel handiger om van gezichtspunt te veranderen. Als ik dat doe, en de evolutie even vergeet en de dingen in het hier en nu bekijk, komt het teleologische woordgebruik weer boven: de ZIN van de kasteverdeling en de DOELMATIGHEID van de signalen. Dit gebeurt niet alleen wanneer ik spreek over mierenkolonies, maar ook wanneer ik nadenk over mijn eigen hersenen en de hersenen van anderen. Maar als dat nodig is kan ik me, zij het met enige moeite, altijd wel het andere standpunt indenken en ook al deze standpunten hun betekenis ontnemen.

Schildpad: Kun je het volgende beantwoorden, dr. Miereneter? Bestaat een signaal, vanaf zijn ontstaan tot aan zijn ontbinding, altijd uit dezelfde verzameling mieren?

Miereneter: Inderdaad haken de individuen in een signaal soms af en als er enkele in het gebied zijn worden ze vervangen door andere van dezelfde kaste. Meestal bereiken signalen de plaats waar ze uiteenvallen met andere mieren dan bij het begin.

Kreeft: Ik begrijp dat de signalen constant de kasteverdeling in de kolonie beïnvloeden, al naar gelang de interne behoeften van de kolonie - die op hun beurt weer de externe situatie weerspiegelen waarmee de kolonie wordt geconfronteerd. Zodoende past de kasteverdeling, om met jouw woorden te spreken, dr. Miereneter, zich voortdurend aan de actuele stand van zaken aan, op een manier die uiteindelijk de buitenwereld weerspiegeld.

Achilles: Maar hoe zit het dan met die tussenliggende structurele niveaus? Je beweerde dat de kasteverdeling beter niet kon worden afgeschilderd in mieren of signalen, maar in termen van ploegen waarvan de leden weer andere ploegen vormen, enzovoort, tot je op miere-niveau komt. En je zei dat dat de sleutel was tot de vraag hoe de kasteverdeling

te beschrijven als gecodeerde stukjes informatie over de wereld.

Miereneter: Ja, daar komen we nog op terug. Ik geef er de voorkeur aan ploegen van voldoende hoog niveau 'symbolen' te noemen. Maar bedenk wel, deze betekenis van het woord verschilt aanzienlijk van de normale betekenis. Mijn 'symbolen' zijn ACTIEVE SUBSYSTEMEN en ze zijn samengesteld uit actieve subsystemen van een lager niveau ... Ze verschillen daarom ook sterk van PASSIEVE symbolen buiten het systeem, zoals de letters van het alfabet of muzieknoten, die onbeweeglijk op hun plaats blijven in afwachting van het moment waarop een actief systeem ze verwerkt.

Achilles: O, dit is wel erg ingewikkeld hè? Ik had er geen idee van dat een mierenhoop zo'n abstracte structuur had.

Miereneter: Ja, dat is heel bijzonder. Maar al deze structurele lagen zijn nodig om het soort kennis op te slaan dat een organisme in staat stelt 'intelligent' te zijn in iedere - redelijke - zin van het woord. Ieder systeem dat een taal vormt, bezit in wezen dezelfde onderliggende verzameling van niveaus.

Achilles: Hola! Wil je daarmee suggereren dat mijn hersenen in wezen bestaan uit een zootje rondrennende mieren?

Miereneter: Nee, dat niet direct. Je moet het niet zo letterlijk opvatten. Het laagste niveau kan geheel verschillend zijn. De hersenen van miereneters zijn ook niet samengesteld uit mieren. Maar als je een niveau of twee omhoog gaat in de hersenen, bereik je een niveau met elementen die hun volmaakte tegenhangers hebben in andere systemen van gelijk intellectueel niveau -zoals mierenkolonies.

Schildpad: Daarom is het ook redelijk, Achilles, om jouw hersenen af te beelden op een mierenkolonie en niet op de hersenen van zomaar een mier.

Achilles: Bedankt voor het compliment. Maar hoe moet je je zo'n afbeelding voorstellen? Wat correspondeert in mijn hersenen bijvoorbeeld met de ploegen op een lager niveau die jij signalen noemt?

Miereneter: O, op hersengebied ben ik maar een amateur en daarom zou ik de afbeelding niet tot in details kunnen uitdenken. Maar - en waarschuw me als ik het mis heb, meneer Kreeft - ik heb zo'n vermoeden dat bij de hersenen de tegenhanger van het signaal in de mierenkolonie, het afvuren van een neuron is; of misschien is het een grootschaliger gebeurtenis, zoals een patroon van neuraal afvuren.

Kreeft: Daar kan ik wel in meegaan. Maar bij onze discussie gaat het er toch niet in de eerste plaats om de exacte tegenhanger aan te geven, hoe wenselijk dat ook moge zijn? Volgens mij is het het belangrijkste dat er zo'n overeenkomst bestaat, ook al weten we op dit moment nog niet precies hoe we hem moeten definiëren. Ik zou alleen een vraagteken willen zetten bij één punt van jou, dr. Miereneter; het heeft te maken met het niveau waarop je kunt verwachten dat de overeenkomst begint. Jij denkt geloof ik dat een SIGNAAL een directe tegenhanger kan hebben in de hersenen; terwijl ik van mening ben dat het pas vanaf jouw ACTIEVE SYMBOLEN en hoger waarschijnlijk is dat er een overeenkomst bestaat.

Miereneter: Jouw interpretatie kan best veel nauwkeuriger zijn dan de mijne, meneer Kreeft.

Bedankt voor dit subtiele argument.

Achilles: Wat doet een symbool dat een signaal niet doet?

Miereneter: Dat lijkt op het verschil tussen woorden en letters. Woorden zijn, als betekenis dragende entiteiten, samengesteld uit letters, die op zich geen betekenis dragen. Dat geeft een goed idee van het verschil tussen symbolen en signalen. Het is in feite een bruikbare analogie, zolang je maar niet uit het oog verliest dat woorden en letters PASSIEF zijn, en symbolen en signalen ACTIEF.

Achilles: Daar zal ik aan denken, maar ik geloof niet dat ik begrijp waarom het verschil tussen actieve en passieve entiteiten zo ontzettend belangrijk is.

Miereneter: De reden daarvoor is dat de betekenis die je toekent aan een passief symbool, zoals een woord op een bladzij, eigenlijk afkomstig is van de betekenis van corresponderende actieve symbolen in je hersenen. Zodat de betekenis van passieve symbolen alleen goed geïnterpreteerd kan worden als deze in verband wordt gebracht met de betekenis van actieve symbolen.

Achilles: Oké. Maar waaraan ontleent een SYMBOOL - een actief symbool, voor alle duidelijkheid - zijn betekenis, als je stelt dat een SIGNAAL - zelf toch ook een volmaakte entiteit - geen betekenis heeft?

Miereneter: Dat heeft allemaal te maken met de manier waarop symbolen ervoor kunnen zorgen dat andere symbolen worden geactiveerd. Als een symbool actief wordt, geschiedt dit niet in isolement. Het zweeft rond in een medium dat wordt gekarakteriseerd door de kasteverdeling.

Kreeft: Natuurlijk is er in de hersenen niet zoiets als een kasteverdeling, maar de tegenhanger daarvan is de 'staat van de hersenen'. Dan beschrijf je de staat van alle neuronen en alle onderlinge verbindingen en de drempel voor het afvuren van elk neuron.

Miereneter: Goed dan: laten we 'kasteverdeling' en 'staat van de hersenen' onder een gezamenlijke noemer brengen en gewoon spreken over de 'staat'. Nu kan de staat op een laag niveau, of op een hoog niveau worden beschreven. Een beschrijving op laag niveau van de mierenkolonie vraagt om een nauwkeurige specificatie van de positie van iedere mier, naar leeftijd, kaste en meer van dergelijke zaken. Een zeer gedetailleerde beschrijving die weinig opheldering verschaft over de vraag WAAROM zij in die staat verkeert. Anderzijds zou bij een beschrijving op hoog niveau gespecificeerd moeten worden welke symbolen door welke combinatie van andere symbolen kunnen worden geactiveerd, onder welke condities, enzovoort.

Achilles: Wat dacht je van een beschrijving op het niveau van signalen, of ploegen.

Miereneter: Een beschrijving op dat niveau zou het midden houden tussen een beschrijving op laag niveau en de beschrijving op symbool niveau. Deze beschrijving zou een grote hoeveelheid informatie bevatten over wat er feitelijk gaande is op bepaalde plaatsen verspreid over de hele kolonie, maar minder dan een mier-voor-mierbeschrijving, want ploegen bestaan uit groepjes mieren. Een ploeg-voor-ploegbeschrijving is net zoiets als een samenvatting van een mier-voor-mierbeschrijving, maar je moet extra gegevens die niet aanwezig waren in de mier voor mier beschrijving toevoegen - zoals de betrekkin-

gen tussen ploegen en de verspreiding van verschillende kasten over diverse plekken. Deze extra complicatie is de tol die je moet betalen voor het recht van samenvatten.

Achilles: Ik vind het interessant om de voordelen van de beschrijvingen op verschillende niveaus met elkaar te vergelijken. Een beschrijving op het hoogste niveau lijkt het meest te kunnen verklaren, omdat deze je het meest intuïtieve beeld van de mierenkolonie geeft, maar vreemd genoeg laat ze het kennelijk belangrijkste kenmerk buiten beschouwing: de mieren.

Miereneter: Het mag er dan anders uitzien, maar de mieren zijn niet het belangrijkste kenmerk. Toegegeven, zonder hen zou de kolonie niet bestaan; maar iets dergelijks, de hersenen, kunnen wel mierloos bestaan. Dus je kunt, op hoger niveau, best buiten de mieren.

Achilles: Ik denk niet dat de mieren jouw theorie in dank zullen afnemen.

Miereneter: Ik ben nog nooit een mier van hoog niveau tegengekomen.

Kreeft: Je schets wel een contra-intuïtief beeld, dr. Miereneter. Als wat je zegt waar is, moet je om de hele structuur te kunnen bevatten in je beschrijving ervan kennelijk iedere verwijzing naar zijn fundamentele bouwstenen weglaten.

Miereneter: Misschien dat ik het je kan verduidelijken met een analogie. Stel je hebt een roman van Charles Dickens voor je.

Achilles: The Pickwick papers - is dat goed?

Miereneter: Voortreffelijk! En stel je nu het volgende spelletje voor: je moet een manier vinden om letters af te beelden op ideëen, zodat de hele Pickwick Papers ook iets zinnigs voorstelt als je hem letter voor letter leest.

Achilles: Hmm... Je bedoelt dat ik iedere keer wanneer ik bijvoorbeeld op het woordje 'het' stuit, aan drie vaststaande begrippen moet denken, het een na het ander, zonder enige ruimte voor variatie?

Miereneter: Precies. Het 'h'-begrip, het 'e'-begrip en het 't'-begrip - en steeds weer zijn deze begrippen hetzelfde als de keer ervoor.

Achilles: Zo te horen wordt het 'lezen' van The Pickwick papers een onbeschrijflijk vervelende nachtmerrie. Het zou een oefening in zinloosheid worden, ongeacht de begrippen die ik met de letters associeerde.

Miereneter: Precies. Er is geen natuurlijke afbeelding van de individuele letters op de werkelijke wereld. De natuurlijke afbeelding vindt plaats op een hoger niveau: tussen woorden en delen van de werkelijke wereld. Als je het boek wilt beschrijven hoef je het letterniveau dan ook niet te vermelden.

Achilles: Natuurlijk niet! Ik zou de plot en de figuren die erin voorkomen beschrijven, enzovoort.

Miereneter: Aha. Je zou dus alle bouwstenen weglaten in je beschrijving, ook al dankt het boek zijn bestaan aan die letters. Ze zijn het medium, niet de boodschap.

Achilles: Akkoord - maar hoe zit het dan met mierenkolonies?

Miereneter: Daarbij heb je actieve signalen in plaats van passieve letters en actieve symbolen in plaats van passieve woorden - maar het idee blijft hetzelfde.

Achilles: Bedoel je dat ik geen afbeelding tussen signalen en dingen in de werkelijke wereld

kan vormen?

Miereneter: Dan zal je ontdekken dat het je niet lukt nieuwe signalen zo te activeren dat ze enige zin hebben. En ook op een lager niveau - bijvoorbeeld op het mierniveau - zal je dat niet lukken. Alleen op symboolniveau hebben de activeringspatronen betekenis. Stel je voor dat je op een dag Myra Hoop observeerde en ik kwam op bezoek. Hoe zorgvuldig je ook observeerde, toch zou je vermoedelijk niets meer waarnemen dan een herschikking van mieren.

Achilles: Dat zal wel.

Miereneter: Toch zou ik, kijkend naar het hogere niveau in plaats van het lagere, zien hoe verschillende slapende symbolen gewekt werden; symbolen die zich laten vertalen in: 'Ach, daar heb je die charmante dr. Miereneter weer - wat aardig! - of woorden van gelijke strekking.

Achilles: Dat lijkt op wat er gebeurde toen we allevier verschillende niveaus vonden om de MU-tekening te lezen - of althans DRIE van ons ...

Schildpad: Wat een ongelooflijk toeval dat er een dergelijke overeenkomst bestaat tussen de vreemde tekening die ik tegenkwam in Wohl Temperierte Clavier en de loop van ons gesprek.

Achilles: Denk je dat het alleen maar toeval is?

Schildpad: Natuurlijk.

Miereneter: Wel, ik hoop dat je nu begrijpt hoe de gedachten in tante Myra Hoop voortkomen uit de symbolen die samengesteld zijn uit signalen die samengesteld zijn uit ploegen die samengesteld zijn uit ploegen op een lager niveau, en steeds verder omlaag, tot aan de mieren.

Achilles: Waarom noem je dat 'symboolmanipulatie'? Wie voert de manipulatie dan uit, de symbolen zijn immers zelf actief. Wie is het agens?

Miereneter: Dat voert ons terug tot de vraag over het doel, die je eerder stelde. Je hebt gelijk als je zegt dat de symbolen zelf actief zijn, maar toch zijn de activiteiten die zij ontplooien niet per definitie vrij. De activiteiten van alle symbolen zijn strikt bepaald door de staat van het complete systeem waarin zij voorkomen. Daarom is het complete systeem verantwoordelijk voor de manier waarop zijn symbolen elkaar activeren en daarom is het alleszins redelijk om het complete systeem te beschouwen als het agens. Je kunt het complete systeem een naam geven. Zo is tante Myra Hoop de 'wie' van wie gezegd kan worden dat ze haar symbolen manipuleert; en voor jou geld hetzelfde, Achilles.

Achilles: Dat is een nogal vreemde karakterisering van de notie wie ik ben. Ik kan het misschien niet helemaal begrijpen, maar ik zal er eens over denken.

Schildpad: Het zou interessant zijn om de symbolen in jou hersenen te volgen terwijl jij nadenkt over de symbolen in jou hersenen.

Achilles: Dat wordt mij te ingewikkeld. Ik heb al moeite genoeg om te bedenken hoe het mogelijk is naar een mierenkolonie te kijken en haar te lezen op symboolniveau. Ik kan me wel voorstellen dat ik haar waarneem op mierniveau; en met een beetje inspanning

kan ik me voorstellen hoe het moet zijn om haar waar te nemen op signaalniveau; maar in hemelsnaam, wat is het om een mierenkolonie waar te nemen op symboolniveau?

Miereneter: Dat leer je alleen door langdurige oefening. Maar als je eenmaal zover bent als ik, lees je het bovenste niveau van een mierenkolonie even makkelijk als je het 'M U' leest in de MU-tekening.

Achilles: Is dat heus? Dat moet wel een verbijsterende ervaring zijn.

Miereneter: In zekere zin wel - maar toch is het ook iets wat jij heel goed kent, Achilles.

Achilles: Goed kennen? Wat bedoel je? Ik heb nog nooit anders naar een mierenkolonie gekeken dan op mierniveau.

Miereneter: Misschien niet, maar in veel opzichten verschillen mierenkolonies niet van hersenen.

Achilles: Maar ik heb nog nooit hersenen gezien of gelezen.

Miereneter: En je eigen hersenen dan? Ben je je dan niet bewust van je eigen gedachten? Is dat niet de essentie van bewustzijn? Wat doe je anders dan je eigen hersenen direct aflezen op symboolniveau?

Achilles: Zo heb ik het nog nooit beschouwd. Bedoel je dat ik voorbijga aan alle lagere niveaus en alleen het bovenste niveau zie?

Miereneter: Zo gaat dat althans bij bewuste systemen. Ze nemen zichzelf alleen waar op symboolniveau en ze zijn zich niet bewust van de lagere niveaus, zoals de signaalniveaus.

Achilles: Betekent dit dat er in de hersenen actieve symbolen zijn die zichzelf constant actualiseren, zodat ze de totale staat van de hersenen zelf weerspiegelen, natuurlijk op symboolniveau?

Miereneter: Jazeker. In ieder bewust systeem zijn er symbolen die de staat van de hersenen vertegenwoordigen en die zelf deel uitmaken van de staat van de hersenen die zij symboliseren. Bewustzijn vergt immers een grote mate van zelfbewustzijn.

Achilles: Dat is een krankzinnig idee. Dus, hoewel er in mijn hersenen de hele tijd een razende activiteit plaatsvindt, kan ik de activiteit slechts op één manier registreren - op symboolniveau; en ik ben volmaakt ongevoelig voor de lagere niveaus. Het is zoiets als het lezen van een roman van Dickens door middel van directe visuele waarneming, zonder ooit de letters van het alfabet geleerd te hebben. Ik kan me niet voorstellen dat zoiets krankzinnigs werkelijk gebeurt.

Kreeft: Maar iets dergelijks gebeurde toen jij 'MU' las, zonder de lagere niveaus 'HOLISME' en 'REDUCTIONISME' waar te nemen.

Achilles: Je hebt gelijk - ik ging voorbij aan de lagere niveaus en zag alleen de bovenlaag. Wellicht mis ik door alleen het symboolniveau te lezen ook allerlei betekenissen op lagere niveaus van mijn hersenen. Jammer dat het bovenste niveau niet alle informatie bevat over het laagste, zodat je door de top te lezen ook ervaart wat het onderste te zeggen heeft. Maar het zal wel naïef zijn om te denken dat in het bovenste niveau iets van het onderste is gecodeerd - het zou vermoedelijk niet naar boven doordringen. De MU-tekening is daarvan het meest frappante voorbeeld dat ik me kan voorstellen: het bovenste niveau luidt daar alleen 'MU', wat in geen enkele relatie staat tot de lagere niveaus!

Kreeft: Dat is volkomen waar. (pakt de MU-tekening op om hem nog eens te bekijken.) Hmm... Er is iets aan de hand met de kleinste lettertjes van deze tekening; ze zijn zeer priegelig...

Miereneter: Laat mij eens kijken. (Tuurt van heel dichtbij naar de MU-tekening.) Ik geloof dat er nog een niveau is dat we allemaal gemist hebben!

Schildpad: Spreek voor jezelf, dr. Miereneter.

Achilles: 't Is niet waar! Laat eens kijken. (Kijkt heel aandachtig.) Ik weet dat jullie dit niet zullen geloven, maar de boodschap van deze tekening staat pal voor onze neus, diep verborgen. Het is maar één woord, dat telkens herhaald wordt, als een mantra - maar wat een belangrijk woord: 'M U'! Wel heb ik ooit! Het is hetzelfde als het bovenste niveau! En niemand van ons had het in de gaten.

Kreeft: Als we jou niet hadden, Achilles zou het ons nooit zijn opgevallen.

Miereneter: Ik vraag me af of het toeval is dat het hoogste en het laagste niveau samenvallen. Of is het doelbewust gedaan door één of andere schepper?

Kreeft: Hoe kom je daar ooit achter?

Schildpad: Ik zou het niet weten, we weten immers ook niet waarom uitgerekend die tekening voorkomt in de uitgave van het Wohl Temperierte Clavier van de Kreeft.

SUMMARY

The central theme of this thesis is the inflammatory interaction between neutrophils and endothelial cells. This inflammatory interaction is regulated by a number of soluble inflammatory mediators, which control the make-up of adhesion molecules on both cell types, and influence their behavior. The investigations presented in this thesis are part of the vast amount of novel information on adhesion molecules and cytokines published during the last decennium. On the basis of this new data, a picture arises in which the molecular basis of the different aspects of neutrophil behavior during inflammation can be defined:

- Selectins, adhesion molecules on both endothelium and neutrophils, mediate neutro-phil rolling by providing rapidly arising and rapidly disappearing highly avid binding sites.
- β_2 integrins on neutrophils guide chemotactic migration by providingdelicately tuned affinity to a broth array of substrata, which increases at the side of the neutrophil where an agonist concentration is sensed exceeding the previous concentration.
- β_2 integrins can, in certain agonist and substrata-conditions, and incollaboration with CD43 on neutrophils, mediate a switch from 'migrating polar phenotype' to the 'immobilized spread phenotype' which is associated with neutrophil toxicity.
- Neutrophil agonists can be divided in 'chemotaxins' and 'secretagogues'. Chemotaxins, such as IL-8, can form gradients, thus supporting transendothelial migration and neutrophil accumulation at inflammatory sites. Secretagogues, such as TNF, support the aforementioned switch from 'migrating polar phenotype' to the 'immobilized spread phenotype' and initiate neutrophil toxicity.

The identification of these mediators, and the definition of their functions, which is depicted in detail in *chapter 1* of this thesis, has been of great benefit in understanding neutrophil behavior. The findings leave us with the challenge of translating this knowledge into clinical benefit for patients.

The experimental work presented in this thesis focusses on TNF, E-selectin, and the role of these molecules in neutrophil endothelial cell interaction. Investigations on these specific subjects are presented respectively in *chapter 3*, *chapter 4* and *chapter 5*.

In *chapter 2*, a short and more specific introduction to, and discussion of the experimental work is given.

In *chapter 3* the conditions which lead to the release of TNF and related cytokines (IL-6 and IL-8) were investigated. Mononuclear phagocytes, activated by lipopolysaccharides from the outer leaflet of gram negative bacteria are the '-classical' origin of soluble TNF during inflammation. TNF is, however, also released in response to other microbial surface components and serum-factors such as complement *(chapter 3.1)*. Furthermore, TNF-release is shown not to be restricted to leukocytes, but to occur also by cytokine-activated renal epithelial cells *(chapter 3.2)*. TNF thus can be associated with a wide array of inflammatory processes, which argues for a more basic role of TNF in inflammation. TNF-release by mononuclear phagocytes in response to lipopolysaccharides, is influenced by two homologous proteins, LPS binding protein, released during inflammation by the liver, and bactericidal/permeability-increasing protein, released by activated neutrophils. These two proteins can respectively increase and inhibit the detection of lipopolysaccharides by mononuclear phagocytes *(chapter 3.3)*. Thus, TNF-release during gram-negative infection seems to be a host regulated event, rather than an overshoot-reaction to a bacterial 'toxin'.

Chapter 4 focusses on E-selectin, an inducible endothelial cell adhesion molecule which facilitates neutrophil 'rolling', the first step in neutrophil endothelial cell interaction during inflammation. Interference with E-selectin expression or E-selectin function thus might be effective in preventing neutrophil mediated inflammatory processes. E-selectin expression by endothelium is induced by the inflammatory mediators TNF and IL-1 and by bacterial lipopolysaccharides. Other factors can enhance E-selectin expression, such as IFN-γ *(chapter 4.2)* and yet unidentified serum factor(s) *(chapter 4.3)*.
Furthermore, recognition of bacterial lipopolysaccharides by endothelial cells was shown to require CD14 *(chapter 4.4)*. Future experiments will be necessary to determine the relevance of interference with E-selectin expression via these pathways. Besides mediating neutrophil rolling, E-selectin might have other functions. E-selectin is not stably expressed, but was found to be internalized rapidly after arriving on the cell-surface *(chapter 4.1)*. The function of E-selectin internalization is unknown. By co-internalization of immune-complexes and soluble adhesion inhibiting factors, which are known to have affinity for

E-selectin, E-selectin might play an important role in decontaminating the circulation in situations of severe immunological challenge.

In *chapter 5*, the involvement of TNF and E-selectin in neutrophil-mediated endothelial cell damage was investigated. In comparison to other physiological neutrophil agonists, TNF showed to be the strongest trigger for neutrophil-mediated endothelial cell injury *(chapter 5.1 and 5.2)*. TNF induced neutrophil mediated endothelial cell injury depended completely on TNF-activation of neutrophils *(chapter 5.1 and 5.3)*, which identifies TNF as a representative of a novel group of neutrophil agonists. Endothelial cells did, however, participate in neutrophil activation by TNF, probably by expressing surface bound PAF in response to initial neutrophil H_2O_2-release (chapter 5.4). TNF was not unique in its capacity to activate neutrophils. FcγRII mediated neutrophil activation, induced by a neutrophil binding anti-elastase monoclonal antibody was able to mimic TNF in its capacity to induce neutrophil respiratory burst activity *(chapter 5.2)*. This mechanism might play a role in the pathogenesis of tissue damage in clinical auto-immune syndromes with circulating elastase-reactive antibodies, such as Wegener's granulomatosis.

TNF-induced neutrophil activation required specific adhesive interactions. The contributions of different adhesion-molecules, and of adhesion-molecule independent neutrophil adherence in TNF-induced neutrophil activation were evaluated. $\beta_2$integrin member CD11b/CD18 mediated substrate-interaction appeared to be essential in neutrophil toxicity incited by TNF *(chapter 5.3)*. Neutrophil CD11b/CD18 thus appears to be more than just an adhesion molecule. It controls the switch to a highly flattened phenotype which adapts the neutrophil to releasing its array of protein degradating enzymes and starting an ongoing burst of reactive oxygen species.

SAMENVATTING

De interactie tussen neutrofiele granulocyten en endotheelcellen tijdens ontstekings-processen staat centraal in dit proefschrift. Deze interactie wordt gestuurd door een aantal ontstekings-mediatoren, die de aanwezigheid van adhesie-moleculen op beide cel-typen reguleren, en het gedrag van deze cellen beïnvloeden.
De onderzoeken in dit proefschrift zijn ingebed in de grote hoeveelheid nieuwe informatie over cytokinen en adhesie-moleculen die de laatste 10 jaar verschenen is. Op basis van deze informatie kan een indeling gemaakt worden van de moleculaire basis van de verschillende fasen van neutrofiel-gedrag tijdens ontstekingen:

- Selectines, adhesie-moleculen die zowel op endotheelcellen als op neutrofielen voorkomen, zijn betrokken in neutrofiel 'rolling' doordat ze neutrofielen kortdurend krachtig aan de vaatwand kunnen verankeren.
- De op neutrofielen aanwezige β_2 integrins zijn van belang bij chemotactische bewegingen, doordat ze, aan die kant van de neutrofiel waar deze een verhoging in de concentratie van een chemotactische stof waarneemt, een gedoseerde affiniteits-toename kunnen teweegbrengen.
- β_2 integrins kunnen samen met CD43 op neutrofielen, wanneer de juiste substraat- en stimulus-condities aanwezig zijn, de neutrofiel laten veranderen van een 'polair migrerend fenotype' naar een 'geïmmobiliseerd uitgespreid fenotype', een vormverandering die in verband staat met neutrofiel-toxiciteit.
- Neutrofiel agonisten kunnen worden ingedeeld in 'chemotaxinen' en 'secretagogen'. Chemotaxinen, zoals IL-8, kunnen gradiënten vormen, die het uit de bloedbaan treden en het migreren naar ontstekings-centra van neutrofielen bewerkstelligen. Secretagogen, zoals TNF, bewerkstelligen de bovengenoemde vormverandering van neutrofielen, en induceren neutrofiel toxiciteit.

De identificatie van deze mediatoren, en het ontrafelen van hun functie, hetgeen in *hoofdstuk 1* van dit proefschrift in detail uitgewerkt is, heeft een grote vooruitgang in het begrijpen van neutrofiel gedrag tijdens ontstekingen gegeven. Daarmee is een nieuwe uitdaging geschapen om dit begrip om te zetten in concrete vooruitgang in therapeutische mogelijkheden bij ontstekings-ziekten.

In het in dit proefschrift beschreven experimentele werk staan TNF, E-selectin en neutrofiel endotheelcel interactie centraal. De onderzoeken over deze specifieke onderwerpen zijn respectievelijk in *hoofdstuk 3, 4 en 5* weergegeven.
Een korte, meer specifieke introductie in, en discussie van het experimentele werk in deze hoofdstukken wordt in *hoofdstuk 2* gegeven.

In *hoofdstuk 3* werden de condities onderzocht, die afgifte van TNF en andere cytokinen zoals IL-6 en IL-8 tot gevolg hebben. Door lipopolysacchariden uit de buitenwand van gram negatieve bacteriën (endotoxinen) gestimuleerde mononucleaire phagocyten, vormen de 'klassieke' bron van TNF tijdens ontstekingsprocessen. De afgifte van TNF en andere cytokinen kan echter ook veroorzaakt worden door andere microbiële oppervlakte-componenten en door serum-factoren zoals complement *(hoofdstuk 3.1)*, terwijl ook andere cellen dan leukocyten, zoals uit de nier afkomstige epitheelcellen, een belangrijke cytokinen-bron bleken te kunnen zijn *(hoofdstuk 3.2)*.
Gezien de veelheid van ontstekings-processen welke met TNF-afgifte gepaard gaan, lijkt een fundamentele rol voor TNF bij ontstekingen waarschijnlijk. TNF-afgifte door mononucleaire phagocyten in aanwezigheid van endotoxinen wordt beïnvloed door twee homologe eiwitten, 'LPS binding protein' afgegeven door de lever tijdens systemische ontstekingen, en 'bactericidal/permeability increasing protein' afgegeven door geactiveerde neutrofielen. Deze eiwitten kunnen de detectie van endotoxinen door mononucleaire phagocyten stimuleren respectievelijk blokkeren *(hoofdstuk 3.3)*. Dit impliceert dat TNF-afgifte gedurende gram-negatieve infecties eerder een door de gastheer gereguleerd fenomeen is, dan een uit de hand gelopen reactie op een bacterieel toxine.

In *hoofdstuk 4* staat E-selectin centraal, een induceerbaar endotheelcel adhesiemolecuul dat neutrofiel 'rolling', de eerste stap in neutrofiel endotheelcel interactie tijdens ontstekingen, mogelijk maakt. Het blokkeren van de expressie of de functie van E-selectin zou zodoende neutrofiel gemediëerde onstekings-schade kunnen voorkomen. E-selectin expressie wordt veroorzaakt door ontstekingsmediatoren zoals TNF en IL-1 en door endotoxine. Andere factoren kunnen E-selectin expressie verhogen, zoals IFN-γ *(hoofdstuk 4.2)* en bepaalde nog ongeïdentificeerde serum bestanddelen *(hoofdstuk 4.3)*. In door endotoxine veroorzaakte E-selectin expressie spelen CD14 receptoren een essentiële rol *(hoofdstuk 4.4)*.
Toekomstig onderzoek zal moeten uitmaken of interventie met E-selectin expressie via één van deze routes zinvol is.
E-selectin zou, naast een rol bij neutrofiel 'rolling', nog andere functies kunnen hebben. E-selectin-moleculen worden niet stabiel tot expressie gebracht, maar bleken na een kort verblijf aan het cel-oppervlak weer geïnternaliseerd te wor-

den. De functie van E-selectin internalizatie is onbekend. Via co-internalizatie van immuun-complexen en adherentie remmende factoren, stoffen waarvan bekend is dat deze affiniteit voor E-selectin hebben, zou E-selectin een belangrijke rol kunnen spelen in het 'schoon houden' van de circulatie tijdens ernstige ontstekingsprocessen *(hoofdstuk 4.1)*.

In *hoofdstuk 5* werd de betrokkenheid van TNF en E-selectin in neutrofiel gemediëerde endotheelcel-schade onderzocht. Aanwezigheid van TNF bleek uitgebreide neutrofiel-gemediëerde endotheelcel-schade te kunnen veroorzaken *(hoofdstuk 5.1)*. TNF bleek krachtiger dan andere fysiologische neutrofiel agonisten in het induceren van H_2O_2 produktie door neutrofielen en van endotheelcel-schade *(hoofdstuk 5.1)*, hetgeen TNF identificeert als een vertegenwoordiger van een nieuwe groep neutrofiel agonisten. Hoewel TNF-activatie van endotheelcellen geen rol bleek te spelen bij TNF-geïnduceerde endotheelcel schade *(hoofdstuk 5.1 en 5.3)*, bleken endotheelcellen wel een actief aandeel te hebben in neutrofiel-activatie door TNF, waarvoor membraangebonden PAF, geïnduceerd op de endotheelcel door blootstelling aan H_2O_2 van de geactiveerde neutrofiel,verantwoordelijk lijkt *(hoofdstuk 5.4)*. Het vermogen van TNF om H_2O_2 produktie door neutrofielen te veroorzaken bleek niet uniek te zijn. Sterke en langdurige zuurstof-radicaal produktie door neutrofielen werd ook veroorzaakt door een neutrofiel bindend anti-elastase monoclonaal antilichaam *(hoofdstuk 5.2)*. Dit mechanisme kan een rol spelen in de pathogenese van weefselschade in klinische auto-immuun syndromen met circulerende elastase-bindende anti-lichamen, zoals in de ziekte van Wegener.

TNF-geïnduceerde neutrofiel activatie bleek afhankelijk te zijn van specifiek oppervlakte-contact. De rol van verschillende adhesie-moleculen, en van adhesie-molecuul onafhankelijke neutrofiel adherentie, in TNF-geïnduceerde neutrofiel activatie werd onderzocht. Substraat-contact via de β_2 integrin vertegenwoordiger CD11b/CD18 bleek onmisbaar te zijn voor neutrofiel toxiciteit geïnduceerd door TNF *(hoofdstuk 5.3)*. Neutrofiel CD11b/CD18 blijkt zodoende meer te zijn dan een adhesie-molecuul. Het controleert de verandering van de neutrofiel van een polaire naar een plat uitgestrekte cel die de neutrofiel lijkt aan te passen aan het vrijmaken van zijn toxische areaal van eiwitsplitsende en zuurstof radicaal producerende enzymen.

DANKWOORD

Het zoeken naar een plaats met ideale condities om je te ontplooien, is wellicht het meest elementaire streven van ieder levend organisme.

Het scheppen van plaatsen met ideale condities om je te begeven op de grenzen van kennis en begrip, is wellicht het meest elementaire streven van de hedendaagse westerse cultuur.

De condities op de plek waar ik drie-en-een-half jaar mocht werken (tweede stoel van links, aan de houten laboratorium-tafel aan het raam op lab 4.1 met uitzicht op de schapen van het BMC, en de 'nieuwe vleugel' van Annadal), en de bijdragen daaraan van Wim Buurman, Trudy Jeunhomme, Jet Leeuwenberg, Gaby Francot, Ingeborg Engelberts, Mark Bemelmans en Mieke Dentener binnen het laboratorium algemene heelkunde, en van Gauke Kootstra en Cees van der Linden daarbuiten, die weerspiegeld zijn in dit proefschrift, heb ik niet alleen ervaren als prettig, maar ook als bijzonder.

PUBLICATIONS

During the course of the experimental studies, which are partially presented in this thesis, the following publications were realized:

1. Von Asmuth, E. J. U., J. G. Maessen, C. J. van der Linden, and W. A. Buurman. 1990. Tumour necrosis factor alpha (TNF-α) and interleukin 6 in a zymosan-induced shock model. ***Scand. J. Immunol. 32:313.***

2. Leeuwenberg, J. F. M., E. J. U. von Asmuth, T. M. A. A. Jeunhomme, and W. A. Buurman. 1990. IFN-γ regulates the expression of the adhesion molecule ELAM-1 and IL-6 production by human endothelial cells in vitro. ***J. Immunol. 145:2110.***

3. Von Asmuth, E. J. U., J. F. M. Leeuwenberg, C. J. van der Linden, and W. A. Buurman. 1991. Tumour necrosis factor-α induces neutrophil mediated injury of cultured human endothelial cells. ***Scand. J. Immunol. 34:197.***

4. Von Asmuth, E. J. U., C. J. van der Linden, J. F. M. Leeuwenberg and W. A. Buurman. 1991. Involvement of the CD11b/CD18 integrin, but not of the endothelial cell adhesion molecules ELAM-1 and ICAM-1 in tumor necrosis factor-α induced neutrophil toxicity. ***J. Immunol. 147:3869.***

5. Smeets, E. F., E. J. U. von Asmuth, C. J. van der Linden, J. F. M. Leeuwenberg, and W. A. Buurman. 1992. A comparison of different substrates for culture of human umbilical vein endothelial cells. ***Biotech. Histochem. 67:241.***

6. Von Asmuth, E. J. U., J. F. M. Leeuwenberg, M. Ceska, W. A. Buurman. 1991. LPS and cytokine-induced endothelial cell IL-6 release and ELAM-1 expression; involvement of serum. ***Eur. Cytokine Net. 2:291.***

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9. Dentener, M. A., V. Bazil, E. J. U. von Asmuth, M. Ceska M., and W. A.Buurman. 1993. Involvement of CD14 in lipopolysaccharide induced tumor necrosis factor-α, interleukin-6 and interleukin-8 release by human monocytes and alveolar macrophages. ***J. Immunol. 150:2885.***

10. Von Asmuth, E. J. U., M. A. Dentener, V. Bazil, M. G. Bouma, J. F. M.Leeuwenberg, and W. A. Buurman. 1993. Anti-CD14 antibodies reduce responses of cultured human endothelial cells to endotoxin. ***Immunol. 80:78.***

11. Dentener, M. A., E. J. U. von Asmuth, G. J. M. Francot, M. N. Marra, and W. A. Buurman. 1993. Antagonistic effects of lipopolysaccharide binding protein and bactericidal/permeability-increasing protein on lipopolysaccharide-induced cytokine release by mononuclear phagocytes. Competition for binding to lipopolysaccharide. ***J. Immunol. 151:4258.***

12. Von Asmuth, E. J. U., M. A. Dentener, M. Ceska, and W. A. Buurman.1994. IL-6, IL-8 and TNF production by cytokine and lipopolysaccharide-stimulated human renal cortical epithelial cells in vitro. ***Eur. Cyt. Network 5:301.***

13. Von Asmuth, E. J. U., J. F. M. Leeuwenberg, and W. A. Buurman. Fcγ receptor-mediated activation of neutrophil H_2O_2-release and of neutrophil mediated endothelial cell damage by a monoclonal antibody against elastase. ***Submitted for publication.***

14. Von Asmuth, E. J. U. and W. A. Buurman. Endothelial cell associated platelet-activating factor (PAF), a co-stimulatory intermediate in tumor necrosis factor-α (TNF-α) induced H_2O_2 release by adherent neutrophil leukocytes. ***J. Immunol. in press.***

CURRICULUM VITAE

Eckhardt von Asmuth werd geboren op 11 september 1962 in Haarlem, als tweede zoon van uit de voormalige DDR gevluchte ouders. Zijn vader werkte als offset-drukker.
In Eindhoven groeide hij op binnen het vijf kinderen tellende gezin, en haalde hij in 1980 het atheneum-B diploma.

Van 1981 tot 1987 studeerde hij geneeskunde aan de Rijksuniversiteit Limburg te Maastricht. Tijdens zijn studie verdiepte hij zich in de psychiatrie, de antroposofische geneeskunde en in het studenten-leven. Na zijn artsexamen werkte hij tot einde 1991 aan een door de Nederlandse Nierstichting gesubsidieerd onderzoek naar de rol van cytokinen bij de transplantaat-rejectie. Dit onderzoek, dat uitmondde in de totstandkoming van dit proefschrift, werd verricht op het laboratorium algemene heelkunde in het Biomedisch Centrum van de Rijksuniversiteit Limburg, onder leiding van Dr. W.A. Buurman, en supervisie van Prof. Dr. C.J. van der Linden en Prof. Dr. G. Kootstra.

In 1992 en 1993 volgde hij de Maastrichtse opleiding tot huisarts. Momenteel woont hij samen met zijn vader, zijn vrouw en hun vier zoons in Den Haag, waar hij sinds november j.l. samen met José Korte een antroposofische huisartsenpraktijk voert.