Studies on tumor necrosis factor : endogenous mediators of sepsis and cachexia

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Studies on

TUMOR NECROSIS FACTOR

ENDOGENOUS MEDIATORS OF SEPSIS AND CACHEXIA

STUDIES ON TUMOR NECROSIS FACTOR

Proefschrift

ter verkrijging van de graad van doctor
aan de Rijksuniversiteit Limburg te Maastricht,
op gezag van de Rector Magnificus, Prof. Dr. F.I.M. Bonke,
volgens het besluit van het College van Dekanen,
in het openbaar te verdedigen
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*"Education is experience,
and the essence of experience is self-reliance."*

T.H. White, *The once and future King*.

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INTRODUCTION

GENERAL INTRODUCTION

The role of cytokines in inflammation and immunity

Invasion of an organism by pathogenic organisms or substances evokes an immune response directed at eliminating the pathogen or preventing its detrimental effects on the organism. The inflammatory and immune processes involved in such a response require a complex interplay between different immunocompetent cells, which are often located at sites far away from the intruding pathogen. The recruitment of these cells and the orchestration of their complex activities is effected by hormone-like substances secreted by immune cells in response to the invading pathogen. These hormone-like molecules, which have been given the general name **cytokines**, not only serve regulatory purposes, but also function as effector molecules in the defence against the invading pathogen. Cytokines can exert their regulatory effects on the same cells which synthesize them (autocrine regulation), on cells in the immediate vicinity (paracrine regulation), or, after entering the circulation, on cells at sites different from the site of their production (endocrine regulation). The regulatory effects of cytokines are not restricted to immunocompetent cells, which are mainly of hemopoietic lineage. The function of cells of many other tissues and organs, like vascular endothelial cells, hepatocytes, connective tissue cells, muscle cells, and adipocytes is affected by cytokines as well.

Many different cytokines have already been characterized, and the list of known cytokines is still increasing steadily. The following groups of cytokines are currently discriminated: the largest group is formed by the interleukins, at this time comprising seven cytokines named interleukin 1 to 7 (IL-1 to IL-7). These cytokines are involved in immunoregulatory processes, generally enhancing cellular immune responses. Another group is formed by the interferons, consisting of interferon- α (IFN- α), interferon- β (IFN- β), and interferon- γ (IFN- γ). These cytokines have virostatic properties and enhance various immune responses. Cytokines enhancing the maturation, differentiation, and clonal expansion of hemopoietic progenitor cells are named colony-stimulating factors (CSF). This group consists of the following cytokines: the multi-colony-stimulating factor CSF-1, the granulocyte colony-stimulating factor G-CSF, the macrophage colony-stimulating factor M-CSF, and the granulocyte-macrophage colony-stimulating factor GM-CSF. Finally, two cytokines have been identified primarily

on the basis of their ability to induce necrosis of experimental tumors, and are therefore named tumor necrosis factors. They consist of tumor necrosis factor- α (TNF), also known as cachectin, and tumor necrosis factor- β , also known as lymphotoxin (LT).

Most cytokines are polypeptides, which has made it possible to synthesize them on a large scale using recombinant DNA technology. The availability of recombinant cytokines has greatly facilitated their use for laboratory investigations, which has resulted in many new insights in their role in the regulation of inflammatory and immune processes. In the wake of this basic research data have accumulated in recent years implicating several cytokines as host mediators involved in the pathogenesis of various human diseases like autoimmune diseases, immunodeficiency states, graft-vs-host disease, cancer cachexia, and sepsis. TNF/cachectin has been one of the cytokines strongly believed to be involved in the pathogenesis of several human disease states, especially sepsis.

Isolation of tumor necrosis factor

A spontaneous regression of malignant tumors has occasionally been observed in cancer patients suffering from severe infections. At the end of the previous century the American physician Coley attempted to mimic these empirical findings by administering killed bacteria (Coley's toxin) to cancer patients, with variable results. Toxin therapy enjoyed some popularity in the nineteen thirties, but soon disappeared after the emergence of radiotherapy and chemotherapy. However, scientists remained interested in the mechanisms underlying this phenomenon. Carswell and Old isolated a serum factor present in BCG-infected mice after injection of endotoxin, which caused hemorrhagic necrosis of transplanted meth A sarcomas in these animals. They named this factor **tumor necrosis factor (TNF)** (1). It was a product of endotoxin-treated macrophages and was soon discovered to be present in other species too (2,3). Human TNF was isolated and characterized by Aggarwal and co-workers (4) and its gene was cloned and brought to expression by the same group (5). The availability of recombinant TNF (rTNF) intensified the scientific interest in its role in immune processes in the following years.

Isolation of cachectin

Other investigators, grouped around Cerami at the Rockefeller University, were interested in the mechanism underlying the severe cachectic state accompanying chronic parasitic infection in animals. They observed that parasitic infections in laboratory animals leading to cachexia were accompanied by a marked hypertriglyceridemia. This proved to be caused by a suppression of the enzyme lipoprotein lipase (LPL), which is responsible for the uptake of triglycerides in adipose tissue (6). Suppression of LPL was also found in mice injected with endotoxin and it was shown that a humoral mediator released by macrophages upon stimulation with endotoxin was responsible for the observed inhibition of LPL (7). Moreover, a cachectic state could be induced by administering conditioned medium from endotoxin-treated murine macrophages to healthy mice (8). The humoral mediator responsible for the observed inhibition of LPL was believed to be responsible for the cachexia as well, and

was therefore named **cachectin** by these investigators. Cachectin was purified and proved to be a protein with a molecular weight of 17 kDa, which was produced by macrophages and macrophage-like cell lines after contact with endotoxin (9). After its N-terminal amino acid sequence was determined it became clear that cachectin was identical to the tumor necrosis factor isolated by Carswell and Old (10).

Structure and production of TNF/cachectin

TNF/cachectin is a 17 kDa, non-glycosylated protein secreted primarily by monocytes and macrophages upon stimulation with endotoxin. Its gene is located on the short arm of chromosome 6 in man, and on chromosome 17 in the mouse, in close proximity to the major histocompatibility complex genes (11,12). The TNF gene is closely linked to the gene coding for a structurally and functionally related cytokine, lymphotoxin (LT) or TNF- β , which shows 30% sequence homology to TNF, and binds to the same receptor as TNF.

A remarkable feature of the TNF gene and of several other genes coding for inflammatory mediators (e.g. LT, colony-stimulating factor, interleukin 1, fibronectin, and most interferons) is the presence of a repetitive oligonucleotide sequence (TTAT-TTAT) at the 3'-untranslated region of the gene, which seems to impart instability to the mRNA transcribed from these genes (13). This probably constitutes a built-in protective mechanism limiting production of these highly potent inflammatory mediators.

The regulation of endotoxin-induced TNF production has been studied both in murine and in human systems. In resting murine macrophages, a constant pool of TNF mRNA is present, which is not translated to TNF protein due to posttranscriptional inhibition. Endotoxin enhances TNF mRNA transcription and induces TNF mRNA translation (14). In resting human monocytes, no TNF mRNA can be detected. Upon stimulation of monocytes TNF mRNA is transcribed, and TNF protein is translated (15). TNF production by human monocytes as well as murine macrophages is enhanced by the T cell lymphokine interferon- γ (IFN- γ) (16-18). In murine macrophages IFN- γ stimulates TNF gene expression both at transcriptional and posttranscriptional level (19,20). Besides endotoxin other compounds have been shown to be capable of inducing monocyte TNF production. For example, protein kinase C activating agents strongly stimulate TNF production (15,21). Furthermore, prostaglandin E2 (22), vitamin D3 (23), the complement protein C5a (24), and the colony-stimulating factors GM-CSF and CSF-1 (25,26) have all been shown to enhance TNF synthesis. Studies investigating the intracellular events regulating TNF expression have demonstrated that the second messenger cGMP initiates TNF production, whereas cAMP has an inhibitory effect on TNF production (27,28).

Although it was initially believed that TNF was exclusively produced by cells of the monocyte/macrophage lineage, it has now become clear that several other mononuclear cells, like T lymphocytes, natural cytotoxic cells, and natural killer cells, have the capacity to synthesize this cytokine upon adequate stimulation (29-31). TNF synthesis by these cells, however, is not induced by the agent classically used to trigger monocyte/macrophage TNF production, e.g. endotoxin. Effective stimuli are protein kinase C activating agents like phorbol diester, Ca²⁺-ionophores, and, in the case of T

lymphocytes, also anti-CD3 mitogens in combination with interleukin 2 (32). Expression of the TNF gene has also been demonstrated in B cell lines and in several non-hemopoietic tumor cell lines (33-36). A remarkable finding of these studies is that tumor cell lines producing TNF are themselves resistant to the cytotoxic effects of TNF.

The kinetics of endotoxin-induced endogenous TNF production has been studied in several species. In rabbits, nanomolar concentrations of TNF were detected in the serum after injection of endotoxin. Peak levels were reached about 2 hr after endotoxin administration. TNF was rapidly cleared from the circulation with an approximate half-life of 6-7 min (37). In baboons, peak serum levels of TNF, averaging about 20 ng/ml, were found between 1.5 and 2.5 hr following infusion of live *E. coli*. Serum TNF became undetectable after 4-6 hr (38). In man, TNF could be detected at low levels (ranging between 35 and 730 pg/ml) in the plasma 90-180 min after endotoxin administration. It was rapidly cleared from the circulation, being undetectable 4 hr after endotoxin infusion (39). These studies indicate that TNF is released in a burst-like manner, after which it is rapidly cleared from the circulation. In humans who were given recombinant TNF (rTNF) intravenously as an antitumor agent in phase I clinical trials, an approximate half-life of 15-30 min was calculated (40-42). The organ systems involved in the clearance of TNF in man are currently still unknown.

Biological effects of TNF

Cytotoxic and antitumor effects

TNF exerts its effects on cells through binding with a specific receptor, which is present on many different cell types. The cell membrane expression of this receptor is enhanced by interferon- γ (43,44) and down-regulated by protein kinase C activating agents (45-47). One of the best studied effects of TNF is its cytotoxic activity against various tumor cell lines (48). Only cell lines possessing TNF receptors are sensitive to the cytotoxic activity of TNF. However, cytotoxicity is not solely dependent on the presence of TNF receptors, since many cell lines which are TNF-resistant express TNF receptors in equal amounts as TNF-sensitive cell lines (49-51). Apparently, the cytotoxic effect of TNF on different cells is controlled both at receptor and post-receptor level.

On the basis of its cytotoxic activity TNF is considered to be an important mediator of monocyte/macrophage cytotoxicity against tumor cells (52-56). It may thus constitute a key cytokine involved in the immune surveillance against tumors. Besides its *in vitro* cytotoxicity against tumor cells, TNF is known to induce hemorrhagic necrosis of several tumors *in vivo* (1,57,58). It has also been demonstrated that TNF is the endogenous mediator responsible for the necrosis of experimental tumors induced by endotoxin (59). The *in vivo* antitumor activity of TNF however is not a result of its cytotoxic properties, but is caused by an effect of TNF on tumor vasculature, and requires the presence of a normally functioning T lymphocyte system (60,61). The cytotoxic and antitumor characteristics of TNF has prompted clinical research investi-

gating its value as an anticancer agent. Several phase I studies of human rTNF have already been reported in the literature (40-42,62). The clinical efficacy of rTNF and the types of cancer responsive to its antitumor effects are however currently still unknown.

A phenomenon morphologically closely resembling the hemorrhagic necrosis of implanted tumors after injection of endotoxin or TNF is the Schwartzman reaction. This reaction can be elicited in rabbits and mice after subcutaneous injection of endotoxin, followed 24 hr later by a local or systemic injection of endotoxin, after which a hemorrhagic necrosis of the skin at the injection site occurs. In rabbits it is possible to replace endotoxin by a combination of TNF and IL-1 to prepare skin for a hemorrhagic necrosis after injection of endotoxin 18 hr later (63). In mice, local injection of human rTNF into a skin site which has been prepared by prior subcutaneous injection of endotoxin also induces hemorrhagic necrosis of the injected skin (64). These studies point to a role for TNF in the microvascular injury accompanying infectious disease and sepsis. In this connection it is relevant that TNF has strong thrombomodulatory influences on endothelial cells *in vitro*. TNF enhances procoagulant activity while suppressing production of the anticoagulant protein C, thus favouring clot formation (65). Furthermore, TNF has been shown to induce fibrin formation in intratumoral blood vessels (66).

Immunomodulatory effects

Besides its cytotoxic and antitumor effects TNF has been shown to possess various immunomodulatory properties. Most of these have been discovered by *in vitro* investigations. TNF enhances inflammatory and immune processes in many different ways. It directly activates polymorphonuclear leukocytes (67,68), it enhances T cell responses (69-71), and modulates B cell differentiation (72-75). It stimulates chemotaxis of immunocompetent cells, and increases their binding to endothelial cells (76-79). TNF also modulates the expression of several cell membrane antigens involved in inflammatory and immune responses on different cell types. It differentially regulates expression of major histocompatibility antigens (80,81). TNF is capable of inducing expression of the IL-2 receptor (82), it induces expression of an activation antigen on endothelial cells (83,84), and enhances expression of complement receptors on neutrophils (85). Besides these cellular effects TNF increases production of several inflammatory mediators like platelet activating factor (86-87), prostaglandins, and collagenase (88). Furthermore, the production of several cytokines like interleukin 1 (89,90), interleukin 6 (91), interferon- β 1 (92,93), and the colony-stimulating factors GM-CSF and CSF-1 (94,95), is enhanced by TNF. Many of the immunomodulatory effects of TNF are mediated indirectly, via the induction of other cytokines. For example, it has been demonstrated that the induction of MHC class I antigens by TNF is mediated by interferon- β 1 (93). TNF is a protein characteristic of the acute phase response, having strong pyrogenic characteristics (96,97). Furthermore, TNF has been shown to induce production of several other acute phase proteins *in vivo* (98). Finally, TNF is probably an important effector cytokine in the immune response against viral and parasitic infections, since it has been shown to possess both antiviral (99-101) and antiparasitic (102) characteristics.

Hormonal and metabolic effects

The hormonal and metabolic effects of acute TNF administration have been studied both in experimental animals, and in man. In rats, administration of human rTNF is accompanied by a hyperlipidemia due to inhibition of lipoprotein lipase, preventing uptake of lipids in peripheral adipose tissue, and a stimulation of hepatic lipogenesis (103). Furthermore, hepatic amino acid uptake greatly increases, serum trace metal concentrations decline, and levels of several stress hormones increase following TNF administration (104,105). All of these changes are characteristic of the acute phase response, which has led the authors of the referred articles to speculate that TNF constitutes a key mediator of the acute phase response following trauma, sepsis, or burns. In man, acute administration of rTNF was found to be accompanied by a spectrum of hormonal and metabolic changes very much alike those found in rats (106). Furthermore, the same response was observed when endotoxin was administered to humans instead of TNF, indicating that TNF probably constitutes a key mediator of the acute phase response following acute infections and sepsis (107).

In summary, tumor necrosis factor is a cytokine characteristically produced during an acute phase response, with a broad range of immunoregulatory and inflammatory properties, which is likely to play an important role in the orchestration of host defence mechanisms against infectious and malignant diseases.

THE ROLE OF TNF/CACHECTIN IN HUMAN DISEASE

Besides its physiological role in inflammatory and immune processes depicted in the previous section a large body of literature has accumulated in recent years implicating TNF/cachectin as a host mediator involved in the pathogenesis of several human disease states. Investigations by Cerami and co-workers, exploring the mechanisms underlying the metabolic derangements and cachexia accompanying chronic parasitic infections, have actually led to the discovery of cachectin, which later proved to be identical to tumor necrosis factor. Cachectin was believed to be the crucial mediator of **cachexia** on the basis of its observed effects on lipid metabolism, e.g. it strongly inhibits the expression of the enzyme lipoprotein lipase by adipocytes, thereby preventing uptake of lipids by these cells. This phenomenon was held responsible for the hyperlipidemia and wasting diathesis characteristically accompanying (chronic) infectious diseases and cancer (108-110). Furthermore, mouse macrophages were shown to produce a mediator upon stimulation with endotoxin causing cachexia in mice, and cachectin was implicated as the causative macrophage product (8). The most convincing evidence implicating TNF/cachectin as a 'cachectic' protein came from a study demonstrating that a chinese hamster ovary tumor (CHO) transfected with the human TNF gene, thus continually secreting TNF, caused severe cachexia upon inoculation in mice, whereas non-transfected CHO cells did not (111). However, by lack of pair-fed controls in this study, it remained unclear whether the observed cachexia was caused by a true catabolic effect of TNF/cachectin or whether it was merely a consequence of reduced intake of food and water observed in mice with TNF-producing tumors. Several other studies have questioned the role of TNF/cachectin as mediator of

cachexia. Administration of human rTNF to experimental animals causes a short-lived repression of food and water intake, without causing true cachexia (112-114). In man, no cachexia is observed upon repeated administration of rTNF (41). Furthermore, two elegant studies have demonstrated that probably not TNF, but another, as yet undefined endotoxin-induced macrophage product is responsible for cachexia in a rat study (97,115). Therefore, although TNF/cachectin ranks first in a row of mediators held responsible for cachexia accompanying chronic infections or malignancy, final experimental proof is still lacking as to this day. Furthermore, it has as yet not been possible to detect circulating TNF in humans suffering from severe cachectic states. One study has demonstrated circulating TNF in cancer patients (116). However, it should be questioned seriously whether, in view of the unusually high levels of TNF reported in this study, truly TNF rather than a cross-reactive serum protein was detected in this study.

Besides its role in cachexia TNF is believed to be a key mediator involved in the pathophysiology of sepsis (117). During sepsis, circulating bacteria or bacterial compounds cause a complex pattern of derangements of host physiology, resulting in fever, shock, acidosis, cardiorespiratory insufficiency, renal failure, and widespread damage to other organ systems (118). In recent years data have accumulated indicating that the host derangements observed during sepsis are not caused directly by the infectious agents, but rather by endogenous mediators produced by cells of the immune system in response to overwhelming infection. TNF is strongly believed to be one of these mediators involved in the pathogenesis of sepsis. Its production is induced by bacterial compounds, most notably lipopolysaccharides from gram-negative microorganisms (endotoxin), in all species studied, both in vitro and in vivo (1,37,39,54). Administration of rTNF to experimental animals has been shown to result in pathophysiological changes almost identical to those observed during endotoxic shock states (64,119-121). Moreover, in several species it has been possible to prevent mortality during experimental sepsis by prior administration of neutralizing anti-TNF/cachectin antibodies (38,122,123). In man, circulating TNF was detected shortly after administration of endotoxin to healthy volunteers (39), and the changes observed after administration of rTNF to humans are very much alike those seen during endotoxemia (107). Circulating TNF has also been demonstrated during sepsis in man (124,125, and chapter 8 of this thesis). In these studies a positive correlation was found between the presence of circulating TNF and mortality. These studies substantiate the involvement of TNF in the pathogenesis of sepsis. However, it remains to be determined whether its role is of a causative nature, in which case intervention studies with neutralizing anti-TNF antibodies in human sepsis would be justifiable. Finally, it remains to be mentioned that besides TNF other inflammatory mediators like interleukin 1, and platelet-activating factor are likely to be involved in the cascade of events resulting in sepsis as well (126-130).

Since TNF constitutes a potent inflammatory mediator with cytotoxic potential, numerous investigators have been interested in its possible involvement in the pathology of autoimmune diseases. In vitro studies demonstrated that TNF induces resorption of bone and cartilage and enhances production of prostaglandins by synovial cells (88,131-133). Moreover, we have demonstrated that TNF production by human mono-

cytes is strongly induced by immune complexes (see chapter 4 of this thesis). TNF is therefore suspected to be in part responsible for the tissue destruction and pain accompanying rheumatoid arthritis. It has indeed been possible to demonstrate the presence of TNF in synovial fluid of patients with rheumatoid arthritis (134,135). Besides TNF, other inflammatory mediators like interleukin 1 and interferon- γ are likely to be involved in the pathology of rheumatoid arthritis as well (136,137). TNF has also been implicated as a pathogenetic factor in such diverse autoimmune diseases as lupus nephritis (138), autoimmune vasculitis (139), autoimmune thyroiditis (140), and diabetes mellitus (141,142). Besides in autoimmune diseases, TNF is also suspected to contribute to the pathologic changes accompanying several other diseases. For example, the pathologic changes observed during experimental malaria have in part been attributed to TNF, produced in the course of the anti-parasitic immune response (143). Another study showed that the skin and gut lesions accompanying experimental graft-vs-host disease in mice could be prevented by prior immunization with anti-TNF antibodies (144). During allograft rejection, raised serum levels of TNF have been detected in man, suggesting that TNF is involved in the inflammatory processes leading to rejection of the grafted organ (145).

In conclusion, besides its physiological role as inflammatory and immunoregulatory mediator, TNF/cachectin is strongly implicated as a pathogenetic factor involved in the derangements in host physiology and metabolism and tissue destruction accompanying several, both infectious and non-infectious, diseases.

INTRODUCTION TO AND DISCUSSION OF THE EXPERIMENTS

The cytokine TNF/cachectin constitutes a pivotal mediator of the host response to infection. It has numerous immunoregulatory and inflammatory properties. TNF production is strongly induced by endotoxin. TNF is considered to be the prime endogenous mediator of the detrimental effects of endotoxin. TNF is therefore believed to contribute to the pathogenesis and pathophysiology of multiple organ failure and shock during sepsis (117-119). TNF is also implicated as a mediator in part responsible for the catabolic state accompanying infectious and malignant disease, referred to as cachexia (8,111). Besides its role in the derangements of host physiology and metabolism in sepsis and cachexia, evidence has accumulated in recent years that TNF also contributes to the tissue destruction accompanying several non-septic inflammatory diseases like allograft rejection (145), graft-vs-host disease (144), and autoimmune diseases (138-142). Until recently, endotoxin constituted the only known stimulus of TNF production by monocytes and macrophages. In view of the possible involvement of TNF in various human disease states, the cellular processes regulating its production and secretion were studied in this thesis. Since monocytes constitute the primary cellular source of TNF in man, *in vitro* studies were conducted predominantly with purified monocyte cultures. *In vitro* studies investigating the regulation of TNF production and secretion by human monocytes are described in chapters 3 to 6. Chapters 7 and 8 contain studies on the role of circulating TNF in two different patient populations.

Both in the *in vitro* and *in vivo* studies TNF concentrations were measured with a TNF-specific enzyme-linked immunosorbent assay (ELISA), developed in our laboratory. In this assay, the TNF concentration of test samples is measured according to the principles of a sandwich-ELISA. In short, TNF molecules present in the test samples are captured by monoclonal anti-TNF antibodies, coated to the bottom of wells of a microtiter plate. After washing away unbound TNF molecules, the number of bound TNF molecules are quantitated by sequential incubation with rabbit anti-TNF antibodies and peroxidase-conjugated goat anti-rabbit antibodies, after which a peroxidase-sensitive substrate is added to the wells. The amount of substrate transformed during the enzyme reaction, giving an estimate of the number of TNF molecules present in the well, is measured by determining the optical density at a fixed wavelength. A standard titration curve is obtained by measuring optical densities at known concentrations of recombinant TNF. An example of such a titration curve, obtained in 100% normal human plasma, is shown in the figure. The data of this figure were kindly given to us by Dr. A. Lawson and Dr. J. Hipkiss from Celltech (Slough, UK). The intra-assay coeffi-

Figure. TNF ELISA: standard titration curve of rTNF. Insert: exploded view of the lower concentrations of rTNF. Percentages indicate intra-assay coefficients of variance (cv), defined as $cv = sd/m$, where m and sd respectively represent the mean and the standard deviation of optical densities obtained at serial measurements of known TNF concentrations during one assay.

coefficients of variance at different TNF concentrations are shown in the figure. The inter-assay coefficient of variance of the TNF ELISA is less than 7%. The lower detection limit is 5-10 pg/ml. Comparable results are found at lower serum or plasma concentrations.

TNF is produced primarily by monocytes and macrophages upon activation with bacterial compounds like endotoxin (14,15). Bacterial products therefore constitute the most likely stimulus of TNF synthesis during infectious diseases. In non-infectious inflammatory diseases in which TNF is believed to be involved, other mechanisms underly the induction of TNF synthesis. Many of these diseases, like for instance allograft rejection or graft-vs-host disease, are accompanied by activation of the T lymphocyte system. It has been shown that T cell mitogens like lectins (146), or anti-CD3 monoclonal antibodies (147), induce TNF synthesis by peripheral blood mononuclear cells (PBMC). The influence of two different T cell mitogens, the lectin PHA and the anti-CD3 monoclonal antibody OKT3, on the secretion of TNF by PBMC was studied in chapter 3. Both mitogens dose-dependently induce TNF secretion by PBMC. TNF secretion is shown to correlate with and to be dependent on

proliferation of T lymphocytes. Although others have demonstrated the capacity of T lymphocytes to produce TNF upon activation (29), in the experiments described in chapter 3 TNF is produced predominantly by monocytes. The T cell lymphokine IFN- γ is shown to be an important humoral mediator of T cell-dependent TNF secretion by monocytes. In contrast to murine experiments, in which IFN- γ induces TNF secretion only in the presence of endotoxin (19), IFN- γ -induced TNF secretion by human monocytes is shown to occur in the absence of endotoxin. This study implicates that in many non-infectious diseases accompanied by T cellular activation, like allograft rejection or graft-vs-host disease, local TNF production in inflammatory areas may contribute to the tissue damage. In graft-vs-host disease the involvement of TNF in tissue destruction has indeed been demonstrated (144).

Receptors for the Fc domain of antibodies (FcR) are present on many cells of hemopoietic lineage. On human monocytes, FcR for IgG (Fc γ R) are abundantly present. These receptors link the cellular immune system to the humoral immune system by activating immunocompetent cells upon binding of Fc ligand of antibodies. Fc γ R have been shown to induce secretion of various inflammatory mediators (148-152). Humoral regulation of TNF secretion was studied by investigating the capacity of Fc γ R to induce TNF secretion by monocytes. The results of this study are presented in chapter 4. Cross-linking of Fc γ R, for instance with immune complexes, is shown to strongly induce TNF secretion. This finding has several implications with regard to the role of TNF in humoral immune responses as well as in immune complex diseases. TNF has *in vitro* cytotoxic (53,55), antiviral (99), and antiparasitic (102) properties, suggesting its involvement in immune responses against malignant cells, viruses, or parasites *in vivo*. Generation of TNF during immune responses against malignant cells, viruses, or parasites may well be mediated by Fc γ R on monocytes and macrophages involved in these responses. TNF has been suggested to contribute to the tissue damage observed in immune complex diseases like rheumatoid arthritis (134,135). Immune complex-induced TNF secretion may thus constitute the mechanism underlying the release of TNF in affected joints, leading to local tissue damage.

Two different Fc γ R are constitutively expressed on human monocytes, a 72 kDa high-affinity Fc γ R (Fc γ RI), and a 40 kDa low-affinity Fc γ R (Fc γ RII) (153). The capacity of both Fc γ R to induce TNF secretion by monocytes is studied in chapter 5. Both receptors have been shown to induce other functions of monocytic cells, like superoxide production (154), and cytotoxicity (155). In chapter 5 it is demonstrated that on untreated monocytes, only cross-linking of Fc γ RI leads to TNF secretion. However, after treatment of monocytes with proteolytic enzymes or with the desialylating enzyme neuraminidase, TNF secretion can also be induced by cross-linking of Fc γ RII, apparently due to an increased affinity of this receptor for the cross-linking antibody. A remarkable finding of this study is that TNF secretion can only be induced when Fc γ R are cross-linked through Fc-Fc γ R interactions. Since proteases and neuraminidase are enzymes likely to be present in areas of inflammation, these findings suggest that Fc γ RII functions as a stand-by receptor, activated during inflammatory processes.

Corticosteroids strongly suppress immune and inflammatory processes, for which purpose they are often used in clinical medicine. One important mechanism underlying the immunosuppressive and antiinflammatory effects of corticosteroids is the

inhibition of release of inflammatory mediators by immunocompetent cells (156,157). TNF is believed to be involved in the pathogenesis and pathophysiology of various disease states, many of whom are amenable to treatment with corticosteroids. However, in each of these disease states TNF synthesis is likely to be induced by different pathways. The influence of corticosteroids on the induction of TNF secretion by different stimulatory agents is therefore studied in chapter 6. Corticosteroids are shown to inhibit TNF secretion by monocytes, the degree of inhibition however being dependent on the stimulus used to induce TNF secretion. Strong inhibition is found when TNF secretion is induced by endotoxin or through cross-linking of Fc γ R. This finding may, in view of the involvement of TNF in sepsis and immune complex diseases, in part explain the beneficial effect of corticosteroids in experimental animal sepsis studies (reviewed in 158), and in immune complex diseases like rheumatoid arthritis. Poor inhibition is found when TNF secretion is induced by silica. This may in part account for the absence of a beneficial effect of corticosteroids in pneumoconiosis, a disease state in which various inflammatory mediators, among which TNF (159), may be involved. Finally, only minimal inhibition is found when TNF secretion is induced by the protein kinase C-activating compound PMA. The clinical relevance of this finding remains as yet unclear.

Kidney allograft recipients experiencing acute allograft rejection are frequently treated with antibodies directed against cell membrane antigens of T lymphocytes, as for example with antithymocyte globulin (ATG), or with the monoclonal antibody OKT3. The administration of these antibodies to humans often evokes a vigorous febrile response, accompanied by various other subjective and objective symptoms (160-164). These side effects have been attributed to the release of cytokines from lysed lymphocytes (165). In chapter 3 it has been demonstrated that the anti-T lymphocyte antibody OKT3 induces TNF secretion in cultures of PBMC. Since many of the side effects observed during administration of anti-T lymphocyte antibodies to humans are very similar to those observed after administration of recombinant TNF to humans (40-42), the involvement of TNF in the etiology of side effects of treatment of kidney allograft rejection with ATG was studied. The results of this study are described in chapter 7. It is demonstrated that the first ATG administration is accompanied by a rapid rise in plasma TNF levels. Side effects of ATG treatment are present during the period of raised plasma TNF levels, suggesting that the side effects are, at least in part, caused by TNF. No detectable plasma levels of another pyrogenic cytokine, interleukin 1, were present during ATG therapy. The second ATG dose was neither accompanied by side effects nor by significant plasma levels of TNF, confirming the contribution of TNF in the etiology of these side effects. In vitro studies suggest that ATG-induced TNF release is not a result of lysis or activation of T lymphocytes. Fc receptor-induced TNF secretion by monocytes, as described in chapter 4, may be one of the mechanisms responsible for the release of TNF in these experiments. This study strongly suggests the involvement of TNF in the etiology of adverse reactions during anti-T cell antibody therapy. However, other inflammatory mediators like IL-1, IL-6, IFN- α , and IFN- γ may also be involved.

Numerous experimental animal studies have implicated TNF as a key mediator involved in the pathogenesis of sepsis and septic shock. However, only scarce data are

available on its role in human sepsis, primarily due to the scarcity of assays sensitive enough to measure low levels of circulating TNF. Using a sensitive enzyme-linked immunosorbent assay for TNF, the presence of circulating TNF and its relation to patient outcome was studied in septic patients. The results of this study are shown in chapter 8. In one of four septic patients, circulating TNF can be detected during sepsis. Mortality in these patients is significantly higher than in patients in whom no circulating TNF can be detected. This difference in mortality is not caused by differences in severity of sepsis or multiple organ failure between both patient groups. These findings confirm clinical data from others, demonstrating a correlation between the presence of circulating TNF and mortality in patients suffering from meningococcal sepsis (124, 125). The question whether the presence of circulating TNF during sepsis is merely a prognostic sign indicating poor patient outcome, or whether TNF plays a pivotal role in the etiology of shock and multiple organ failure during sepsis, as has been suggested by results from animal experiments (38,122,123), remains as yet unanswered.

In conclusion, the immunoregulatory and inflammatory cytokine TNF plays a pivotal role in the orchestration of host defence mechanisms against infectious and malignant disease. Besides its physiological role TNF is also implicated as an endogenous mediator contributing to the pathology of various disease states like sepsis, cachexia, autoimmune diseases, graft-vs-host disease, and allograft rejection. In this thesis it is demonstrated that besides the classical pathway of induction of TNF synthesis by bacterial compounds like endotoxin, TNF production and secretion is also likely to be induced during non-infectious inflammatory diseases accompanied by activation of the cellular and humoral immune systems. Furthermore, the presence of circulating TNF is demonstrated in two different patient populations, and the relation between the presence of circulating TNF and the symptomatology and outcome of these patients is discussed.

Undoubtedly, TNF does not act as an isolated mediator during immune responses. It is involved in cascade events, in which a complex interplay between many different inflammatory mediators takes place. For example, TNF initiates synthesis of various other cytokines involved in the acute phase response, like IL-1, IL-6, and platelet activating factor. In its turn, TNF synthesis is regulated by these cytokines, giving rise to complex feedback pathways. Also, physiological and metabolic derangements as observed during sepsis and cachexia are not likely to be caused by one single mediator. Future research has to demonstrate whether interference with the synthesis of single inflammatory cytokines like TNF offers therapeutic opportunities in the treatment of disease states in which these cytokines are believed to be involved.

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T CELL-MEDIATED SECRETION OF TUMOR NECROSIS FACTOR BY MONOCYTES

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SUMMARY

The aim of this study was to examine the production of tumour necrosis factor (TNF)- α by peripheral blood mononuclear cells activated by mitogens. Considerable amounts of TNF, ranging from 1.0 to 5.0 ng/ml, were present in the supernatants of cultures of human peripheral blood mononuclear cells (PBMC), stimulated with either the anti-CD3 monoclonal antibody OKT3 or the lectin phytohaemagglutinin (PHA). The amount of TNF secreted in the supernatant was closely correlated to the degree of T cell proliferation in such cultures, as measured by [3 H]TdR incorporation. In the absence of proliferating T cells the mitogens did not induce secretion of TNF by monocytes. Supernatants of proliferating T cells were shown to induce TNF secretion by monocytes. The macrophage-activating factor gamma interferon (IFN- γ) was also shown to induce, in the absence of endotoxin, TNF secretion by monocytes in a dose-dependent manner. The induction of TNF production by supernatants of *proliferating T cells* could largely be abrogated by passaging the supernatants on an anti-IFN- γ column before adding them to the monocytes. It is therefore concluded from this study that the production of TNF by monocytes can be induced by proliferating T cells and that this induction can largely be attributed to the T cell lymphokine IFN- γ .

INTRODUCTION

Tumour necrosis factor- α (TNF), also called cachectin, is a 17 kDa protein produced and secreted by monocytes and macrophages (4,8,31). In addition to microbial products like lipopolysaccharide (LPS) lectins were also shown to induce TNF production (27), suggesting that TNF release is also under the control of T cell processes, as has been reported for the monokine interleukin 1 (IL-1) (24,25,35). The T lymphocyte product interferon-gamma (IFN- γ) may be involved in this process as a mediator although the capacity of IFN- γ to induce TNF secretion is disputed (27,32,33).

It has recently been shown that not only monocytes but also T lymphocytes and natural killer cells are able to produce TNF after stimulation (11).

In this study we examined whether, in analogy to IL-1, TNF production by monocytes can be regulated by T cell processes. We found that TNF is produced and secreted by monocytes in mitogen-stimulated peripheral blood mononuclear cell (PBMC) cultures and that this production is dependent on the presence of proliferating T cells. It was concluded that TNF production by monocytes can be regulated by T cell processes and that this regulation is largely mediated by the T cell lymphokine IFN- γ .

MATERIALS AND METHODS

Reagents. Human cDNA derived rIFN- γ was kindly provided by Hoffmann La Roche (Basle, Switzerland). It contained about 34 pg LPS per 10^3 IU of IFN- γ , as determined by a chromogenic Limulus amoebocyte lysate assay. rIL-2 was kindly provided for this study by Biogen, Geneva (Dr Galazka). rTNF was kindly provided by Knoll Ag/BASF (Ludwigshafen, FRG), in collaboration with Biogen, Geneva. The specific activity is 8×10^6 lytic units/mg protein in a 48 h L929 assay without mitomycin. Recombinant human GM-CSF (specific activity more than 4×10^7 U/mg protein) was kindly provided by Dr M.A. Cantrell (Immunex, Seattle, Wash., USA). PHA15 (Wellcome, Beckenham, Kent, UK) was used for lectin stimulation of T cells. 4- β -phorbol-12-myristate-13-acetate (PMA), LPS from *Salmonella typhimurium*, and polymyxin B sulphate were purchased from Sigma (St Louis, Mo., USA).

Antibodies. The anti-CD3 monoclonal antibody OKT3 was obtained with the OKT3-secreting hybridoma (CRL8001), purchased from the ATCC (Rockville, Md., USA). Filter-sterilized ascites was used in different dilutions in culture medium to stimulate T cells. Anti-Leu-1, an anti-CD5 monoclonal antibody, was purchased from Becton Dickinson (Mountain View, Calif., USA). The anti-monocyte monoclonal antibody PDV 11-2 was a kind gift from Dr F. Koning (Leiden, The Netherlands). The monoclonal antibody D9D10 against human IFN- γ was kindly provided by Dr M. de Ley (Leuven, Belgium). Monoclonal antibodies against TNF were derived from anti-TNF-secreting hybridomas obtained by a standard cell fusion procedure. Rabbit polyclonal antibodies against TNF were raised by immunizing rabbits with highly purified human rTNF. IgG was semipurified by ammonium sulphate precipitation of the collected serum.

Cell cultures. PBMC were prepared by buoyant density centrifugation on Lymphoprep (Nycomed, Oslo, Norway) of buffy coats of donor blood obtained from the local blood bank. Cells were cultured in 25 cm² culture flasks (Greiner, Nürtingen, FRG), or in 24-well culture plates (Greiner), in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin, and 100 μ g/ml streptomycin (Flow, Irvine, UK), at 37°C in humidified 5% CO₂ and 95% air. The culture medium used in the experiments contained endotoxin levels ranging between 50 and 400 pg/ml. Monocytes were isolated from PBMC by adherence to plastic for 1 h and extensive washing of non-adherent cells and, in some cases, additional removal of remaining T lymphocytes by incubating the cells for 30 min with anti-Leu 1 at 4°C, followed by incubation for 1 h with rabbit complement at 37°C. Monocytes were cultured under the same conditions as the PBMC. Activated T lymphocytes were prepared by isolating the non-adherent cell fraction of PBMC after 24 h of mitogen stimulation and by removing remaining monocytes by treatment with the anti-monocyte monoclonal antibody PDV 11-2 for 30 min at 4°C and rabbit complement for 1 h at 37°C. Proliferation of cells was sustained by adding rIL-2, 10 U/ml, to the cultures.

Proliferation of cells was assessed by incubating 100 μ l of resuspended cells with 0.5 μ Ci [³H]TdR (methyl-[³H]thymidine, Amersham International, Amersham, Bucks., UK; specific activity 5.0 Ci/mmol) in a round-bottomed microtitre plate for 4 h and, after the cells had been harvested with a cell harvester, measuring radioactivity in a liquid scintillation counter. Proliferation was assessed in triplicate.

The purity of the isolated T cells and monocytes was analysed by incubating the cells with the monoclonal antibodies OKT3 and PDV 11-2 for 30 min at 4°C, after which the cells were washed twice and incubated with an FITC-labelled rabbit anti-mouse antibody for 30 min at 4°C. Cells were counted with a FACS IV (Becton Dickinson).

TNF enzyme-linked immunosorbent assay (ELISA). TNF concentrations of cell-free supernatants of the cell cultures were determined with a TNF-specific ELISA. Immunoassay plates (96-well plates; Nunc, Roskilde, Denmark) were coated overnight at 4°C with three monoclonal anti-TNF antibodies at a concentration of 5 µg Ig/ml. Plates were blocked with 1% (wt/vl) BSA in PBS for 1 h at room temperature. After four washes with washing buffer (WB, consisting of PBS, 0.1% BSA, 0.1% Tween 20) test samples were added to the plate for 1 h at room temperature. A standard titration curve was obtained by making serial dilutions of a known sample of rTNF. Next, the plates were washed five times with WB and sequentially incubated with rabbit anti-TNF immune serum, biotinylated anti-rabbit antibody (Amersham) and horseradish peroxidase-labelled streptavidin (Amersham). After adding substrate (o-phenylenediamine, Sigma, 0.43 mg/ml) to the plate for 5-10 min, the colour reaction was stopped with 1.0 M H₂SO₄ and light absorption was measured with a micro-ELISA autoreader (Flow) with a 493 nm filter. The ELISA had a lower detection limit of 50 pg/ml.

Affinity chromatography. In some experiments, supernatants of mitogen-stimulated PBMC were used to induce TNF secretion by monocytes. From these supernatants TNF and IFN-γ were removed by passing the supernatants on a protein A-Sepharose 4B column (Pharmacia Fine Chemicals, Uppsala, Sweden) loaded with a rabbit anti-TNF antibody and a CNBr-activated Sepharose 4B column (Pharmacia) loaded with IgG from the anti-human IFN-γ-producing clone D9D10, respectively. The flow-through fractions did not contain any detectable TNF or IFN-γ after passing on the respective columns, as determined with the TNF ELISA and with titration on Hep-2 cells. Next, these supernatants were dialysed overnight at 4°C against RPMI before adding them to the cell cultures.

Endotoxin measurements. Endotoxin concentrations were measured with a chromogenic Limulus lysate assay purchased from Kabivitrum (Stockholm, Sweden).

RESULTS

TNF production in mitogen-stimulated PBMC

PBMC were stimulated with the anti-CD3 monoclonal antibody OKT3 and with PHA. TNF could be detected in the supernatant of mitogen-stimulated PBMC, sampled after 24, 48, and 72 h, whereas TNF was absent in the non-stimulated cultures (Table I). Since OKT3 and PHA are T cell mitogens, we examined whether T cell proliferation and TNF production are correlated. For this purpose PBMC were incubated with different concentrations of OKT3 and PHA. Fig. 1 gives the dose-response curves for T cell proliferation and TNF production in such mitogen-stimulated PBMC cultures. The figure shows that T cell proliferation and TNF concentration correlate similarly to mitogen concentration.

TNF production in mitogen-stimulated PBMC is T cell-dependent

Since T cell proliferation and TNF production appeared to be correlated, we examined whether TNF production is T cell-dependent or caused by a direct effect of the mitogens on monocytes. Incubation of highly purified monocytes with OKT3 and PHA for 24 h did not lead to a measurable TNF production. TNF production could, however, be induced by LPS and PMA indicating that the monocytes were viable and capable of producing TNF (Table II).

TABLE I. *TNF production in mitogen-stimulated PBMC^a*

	Concentration of TNF- α (ng/ml)			³ H]TdR-uptake(cpm)
	24 h	48 h	72 h	
Experiment 1				
Unstimulated cells	< 0.05 ^b	< 0.05	< 0.05	4869
+ OKT3	3.18	2.97	1.36	51,983
+ PHA	5.64	3.83	2.15	53,127
Experiment 2				
Unstimulated cells	0.08	< 0.05	< 0.05	751
+ OKT3	2.19	1.29	0.72	39,898
+ PHA	4.51	2.58	1.46	70,423
Experiment 3				
Unstimulated cells	< 0.05	< 0.05	< 0.05	854
+ OKT3	0.60	1.00	0.44	28,136
+ PHA	2.10	2.16	2.75	55,390

^a2x10⁶ PBMC were incubated with a 1:500 dilution of OKT3 ascites or a 1:100 dilution of PHA15, and the TNF concentration was measured at 24, 48, and 72 h. Cell proliferation was measured on day 3 of culture. Data from 3 out of 8 different experiments are shown.

^b0.05 ng/ml constituted the lower detection limit of the TNF ELISA.

TABLE II. *Lack of TNF production by isolated monocytes treated with OKT3 and PHA^a*

Treatment	Expt 1	Expt 2	Expt 3
None	< 0.05 ^b	< 0.05	< 0.05
OKT3	< 0.05	< 0.05	< 0.05
PHA	< 0.05	< 0.05	< 0.05
LPS	0.81	1.03	0.34
PMA	2.37	1.93	0.64

^aApproximately 5x10⁵ purified monocytes, obtained by adherence and additional removal of remaining T lymphocytes by complement lysis as described in Methods, were incubated with a 1:500 dilution of OKT3 ascites, a 1:10 dilution of PHA, 10 μ g/ml LPS, and 10 ng/ml PMA, respectively, and the TNF concentration (ng/ml) was measured 24 h later. Data from 3 out of 6 different experiments are shown.

^b0.05 ng/ml constituted the lower detection limit of the TNF ELISA.

Figure 1. Dose-response curves of TNF production and T cell proliferation in mitogen-stimulated PBMC cultures. PBMC at a concentration of 1×10^6 cells/ml were incubated with dilutions of OKT3 ascites (A) and PHA (B). Cell proliferation (open circles) was measured on day 3 of culture and TNF concentration in the supernatant (open squares) was measured after 24 h. The results are means of duplicate cultures and are representative of 10 different experiments.

Next, the influence of T cells on TNF production by monocytes was studied by adding proliferating T cells to isolated monocytes. Table III shows that monocyte-depleted, T cell-enriched cell populations produced only low amounts of TNF, despite persistent cell proliferation sustained by exogenous rIL-2. However, TNF production was greatly increased by the addition of such isolated proliferating T lymphocytes to purified monocytes. To exclude the influence of rIL-2 on monocytes, rIL-2 was added to purified monocytes. The monocytes did not produce TNF after incubation with IL-2 for 24 h (Table III). It was therefore concluded that the mitogens OKT3 and PHA do not have a direct effect on monocytes but exert their effect via activation of T cells.

IFN- γ and GM-CSF both induce TNF production by monocytes

To examine whether a humoral T cell product was responsible for the T cell-mediated TNF production observed, we examined whether the lymphokines IFN- γ and GM-CSF, which are both known inducers of monocyte tumoricidal activity, can induce TNF production by monocytes. Fig. 2 shows that both IFN- γ and GM-CSF induce TNF

TABLE III. *TNF production by T cells and monocytes*^a

Cells	TNF (ng/ml)	[3H]TdR (cpm)
Experiment 1		
PBMC + OKT3	11.5	71,210
Activated T lymphocytes + rIL-2	< 0.05 ^b	29,160
Monocytes + rIL-2	< 0.05	440
Monocytes + activated T lymphocytes + rIL-2	1.31	33,040
Experiment 2		
PBMC + OKT3	2.16	14,970
Activated T lymphocytes + rIL-2	0.68	76,410
Monocytes + rIL-2	< 0.05	920
Monocytes + activated T lymphocytes + rIL-2	4.64	42,380

^a Approximately 1×10^6 activated T lymphocytes (see Materials and Methods) and 5×10^5 monocytes, obtained by adherence and additional removal of remaining T lymphocytes by complement lysis, were incubated with rIL-2, 10 U/ml, and the TNF concentration was measured 24 h later. In the same experiment, 1×10^6 proliferating T lymphocytes were added to 5×10^5 monocytes + rIL-2, 10 U/ml, and the TNF concentration was also measured after 24 h. The TNF concentration in supernatant of unseparated PBMC of the same individual was measured 24 h after induction of mitogenesis with OKT3. Cell proliferation was assessed on day 3 of culture in all experiments. Data from 2 out of 6 different experiments are shown.

^b 0.05 ng/ml constituted the lower detection of the TNF ELISA.

production by purified monocytes in a dose-dependent manner. IFN- γ appeared to be a far more potent stimulus for TNF production than GM-CSF, since the maximal amount of TNF produced by monocytes after incubation with both cytokines is about eight times higher for IFN- γ than for GM-CSF. Both cytokines show an inhibitory effect at higher concentrations.

To exclude the possibility that IFN- γ acts only as a 'primer' of monocytes and that the secretion of TNF is induced by low concentrations of endotoxin present in the culture medium, this medium, containing IFN- γ at different concentrations, was incubated with the endotoxin-neutralizing compound polymyxin B sulphate before adding it to monocytes. The amount of polymyxin B sulphate used, i.e. 1 μ g/ml, was found to inhibit at least 1 ng/ml of LPS (Greve, Buurman *et al.*, unpublished observations), whereas the medium including IFN- γ always contained less than 500 pg/ml of LPS. Fig. 3 shows that there is only a partial reduction of TNF production at the higher concentrations of IFN- γ , while at lower concentrations, TNF production is not inhibited at all. It is therefore concluded that IFN- γ is able to induce TNF production and secretion by monocytes in the absence of endotoxin.

Figure 2. TNF production by monocytes, isolated by adherence and extensive washing, induced by IFN- γ and GM-CSF. Approximately 5×10^5 monocytes were incubated with IFN- γ (open circles) or GM-CSF (open squares), and after 24 h cell-free supernatants were harvested for measurement of TNF concentration. The results are representative of four different experiments.

Figure 3. TNF production by monocytes, induced by IFN- γ in the presence of polymyxin B sulphate. Approximately 5×10^5 monocytes, isolated by adherence and extensive washing, were incubated with IFN- γ (open circles) or with IFN- γ , preincubated for 2 h at 37°C with $1 \mu\text{g/ml}$ polymyxin B sulphate (open squares). After 24 h, cell-free supernatant was harvested for measurement of the TNF concentration. TNF production by monocytes incubated with $1 \mu\text{g/ml}$ polymyxin B sulphate was in this experiment the same as for monocytes incubated with culture medium alone, and averaged 0.15 ng/ml . Data are from one representative experiment out of four different experiments.

Figure 4. TNF production by monocytes induced by supernatant of mitogen-stimulated PBMC. 5×10^6 PBMC were incubated in a 25 cm^2 culture flask with OKT3 1:500 ascites and after 3 days the cell-free supernatant of these cultures was harvested and passed on an anti-TNF column. Part of this supernatant (containing approximately 70 IU of $\text{IFN-}\gamma$) was also passed on an anti- $\text{IFN-}\gamma$ column. Next, this supernatant was added in several dilutions to approximately 5×10^5 monocytes isolated by adherence and extensive washing, and after 24 h samples of supernatant were taken for measurement of the TNF concentration. Open circles represent supernatant that had only been passed on an anti-TNF column. Open squares represent supernatant that had been passed on an anti-TNF column as well as on an anti- $\text{IFN-}\gamma$ column (containing substantially less than 2 IU/ml $\text{IFN-}\gamma$ and less than 50 pg/ml TNF). TNF production by monocytes incubated with 1:500 OKT3 ascites for 24 h as for monocytes incubated with culture medium alone averaged 0.1 ng/ml. Data are from one representative experiment out of five different experiments.

It was also studied whether the GM-CSF-induced TNF secretion was dependent on the presence of low amounts of endotoxin in the culture medium. Preincubation of medium containing GM-CSF at the same concentrations as used in the experiment of Fig. 2 with $1 \mu\text{g/ml}$ polymyxin B sulphate completely inhibited TNF secretion by monocytes (data not shown).

T cell-induced TNF production by monocytes is predominantly mediated by $\text{IFN-}\gamma$

Next, experiments were performed to determine whether $\text{IFN-}\gamma$, produced by proliferating T lymphocytes, is responsible for the T cell-mediated TNF production in mitogen-stimulated PBMC cultures. For this, cell-free supernatants of mitogen-stimulated PBMC were added after 3 days of culture to purified monocytes in several dilutions in culture medium. Before supernatants were added to the monocytes, they were first passed on an anti-TNF column to remove all TNF in order to prevent possible regulatory influences of exogenous TNF. Part of each supernatant was further

Figure 5. TNF production by monocytes induced by supernatant of proliferating T lymphocytes in the absence of mitogen. 5×10^6 PBMC were stimulated with OKT3 1:500 ascites and after 24 h the non-adherent cells were separated from the adherent cells, washed, and treated with the anti-monocyte monoclonal antibody PDV 11-2 and complement to obtain a T cell-enriched cell population. Proliferation of the cells was sustained with 10 U/ml rIL-2. The cell-free supernatant of these cells was harvested after 2 days. After being passaged on an anti-TNF column, and in part also on an anti-IFN- γ column, the supernatant was added in several dilutions to approximately 5×10^5 monocytes isolated by adherence and extensive washing. Open circles represent supernatant that had only been passaged on an anti-TNF column. Open squares represent supernatant that had been passaged on an anti-TNF column as well as on an anti-IFN- γ column. The supernatant of the monocytes was taken 24 h later for measurement of the TNF concentration. Data are from one representative experiment out of four different experiments.

passed on an anti-IFN- γ column. Fig. 4 shows that cell-free supernatants of mitogen-stimulated PBMC induce TNF production by purified monocytes in a dose-related manner. The figure also shows that the TNF production-inducing activity of these supernatants was largely abrogated by passage on an anti-IFN- γ column. To further exclude the role of mitogen in this process the capacity of mitogen-free supernatant of proliferating T cells to induce TNF production by monocytes was studied. Fig. 5 shows that the supernatant of proliferating T cells induces TNF production by monocytes in the absence of mitogen, and that the induction of TNF production was largely abrogated by passage on an anti-IFN- γ column.

DISCUSSION

TNF is a monokine produced by monocytes after stimulation with bacterial LPS (5,9,17,20). The production and secretion of another monokine, IL-1, which resembles TNF in many ways, is induced in a similar way (1,2,21). Furthermore, it is known that IL-1 can be produced by monocytes by a T cell-dependent mechanism (24,25,35). The fact that TNF can be detected in the supernatant of mitogen-stimulated PBMC cultures (27) suggests that there also exists a T cell-dependent mechanism of production for TNF. Our data show that TNF is produced in mitogen-stimulated PBMC cultures, and that the amount of TNF produced correlates with the rate of T cell proliferation in such cultures. This was found both with lectin- and OKT3-induced mitogenesis. A similar phenomenon was observed in two-way mixed lymphocyte cultures (MLC), in which T cell proliferation is induced by alloantigen stimulation (data not shown). It is further shown that TNF is predominantly produced by monocytes. In our system only marginal production by proliferating T cells was observed, indicating that T lymphocytes can produce TNF, as was shown by Cuturi *et al.* (11), who reported that activation of T cells is accompanied by TNF production, especially after activation with phorbol diester and calcium ionophore. The T cell dependence of the mitogen-induced TNF secretion was demonstrated by experiments in which TNF secretion by purified monocytes could be induced by adding proliferating T cells to the monocytes. The data strongly suggest that proliferating T cells induce TNF production by monocytes, although a minor contribution by T cell-derived TNF is not excluded.

The monokines TNF and, to a lesser extent, IL-1 are known to play an important role as mediators of monocyte cytotoxicity (9,13,15,20,28,34). Monocyte cytotoxicity can be regulated by T cell processes. It is predominantly mediated by the production of monocyte-activating factors by T lymphocytes, of which IFN- γ is the most important (12,18,19,22,23,26,29). This lymphokine is produced by mitogen-stimulated, proliferating T cells (14,30). IFN- γ enhances monocyte cytotoxicity by increasing the production of hydrogen peroxide (16,26), and by priming monocytes for the production and secretion of monokines with cytotoxic activity, *i.e.* TNF and IL-1 (2,3,6,10,32). It is generally supposed that for the secretion of these cytotoxic monokines by murine macrophages besides IFN- γ , a second, triggering signal is necessary. This second signal can be provided by bacterial lipopolysaccharide (6,29). Data on the potential of IFN- γ to induce production and secretion of TNF by human monocytes in the absence of LPS are conflicting (27,32,33). In this study we have shown that IFN- γ can induce TNF secretion by human monocytes in the absence of endotoxin. Our data, which differ from those obtained by Nedwin *et al.* (27), but are in agreement with the findings of others (32,33), strongly suggest that human and murine monocytes/macrophages differ with respect to the need for a triggering signal to induce the secretion of TNF.

The experiments show that the TNF-inducing activity of supernatants of mitogen-stimulated PBMC can largely be abrogated by passaging the supernatants on an anti-IFN- γ column. From this it is concluded that the T cell-dependent TNF production that we found in our study is, at least to a great extent, induced by IFN- γ .

It was also found that recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) could induce TNF secretion by monocytes, although it con-

stituted a far less potent stimulus for TNF secretion than IFN- γ . Moreover, the GM-CSF-induced TNF secretion was shown to be dependent on the presence of endotoxin in the culture medium, since it could be totally abrogated by polymyxin B sulphate. This finding is in accordance with those of other investigators (7).

So far it is generally believed that the secretion of TNF by monocytes depends on the presence or absence of bacterial LPS. This study makes it probable that TNF is produced under many conditions in which the immune system is activated.

In conclusion, the results of this study show that in man, TNF is produced by monocytes in mitogen-stimulated PBMC, and that TNF production is dependent on the presence of proliferating T cells. This T cell-dependent TNF production seems to be largely mediated by the T cell product IFN- γ . This finding has potential significance for many disease processes in which the T cell system is involved, for example in the host defence mechanisms against malignancies and viral infections or in the pathophysiology of autoimmune processes like rheumatoid arthritis.

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Fc-RECEPTOR CROSS-LINKING INDUCES SECRETION OF TUMOR NECROSIS FACTOR BY MONOCYTES

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SUMMARY

In this study it was demonstrated that cross-linking of FcR on human monocytes induces the secretion of the cytotoxic and immunoregulatory cytokine TNF. Both soluble and insoluble immune complexes, solid-phase antibody and antibody-coated phagocytizable particles were used to cross-link FcR on monocytes. It was observed that monocytes secreted large amounts of TNF in each of these instances. Kinetic studies performed with soluble immune complexes showed that TNF was secreted very rapidly, e.g. within 2 h after addition of immune complexes to monocytes. These findings are relevant for the understanding of FcR-mediated immune responses by monocytes and macrophages, for example antibody-dependent cellular cytotoxicity and phagocytosis, and for disease states associated with circulating or tissue-fixed immune complexes.

INTRODUCTION

Receptors for the Fc portion of immunoglobulins (FcR) are present on the plasma membrane of most cells of hemopoietic lineage and perform many functions in immune processes. On monocytes and macrophages, FcR are involved in phagocytosis of antibody-coated particles or cells (1, 2), in the mediation of antibody-dependent cellular cytotoxicity (ADCC) (3-7), and in the release of various inflammatory mediators (8-12). FcR are also important for the clearance of circulating immune complexes by cells of the mononuclear phagocytic system (13, 14). Finally, cross-linking of FcR has been shown to trigger the oxidative burst (15, 16).

Tumor necrosis factor (TNF), also known as cachectin, is an important inflammatory mediator produced predominantly by activated monocytes and macrophages

(17, 18). It has cytotoxic properties, both against tumor cells and against normal cells infected with intracellular pathogens and viruses, and performs many immunoregulatory functions, at least in vitro (reviewed in 19).

Because FcR trigger monocyte and macrophage activation and cytotoxicity, we investigated whether the secretion of TNF, which constitutes an important mediator of monocyte/macrophage cytotoxicity, can be induced by FcR cross-linking. The study shows that cross-linking of FcR, both by soluble and insoluble immune complexes, solid-phase antibodies and by antibody-coated phagocytizable particles, induces rapid secretion of TNF by human monocytes. These findings are relevant for the understanding of FcR-mediated monocyte/macrophage functions in immune processes, like ADCC and phagocytosis. The data also suggest that TNF may contribute to the pathophysiology of immune complex diseases, notably rheumatoid arthritis.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA; No. A-6003, essentially fatty acid free), lipopolysaccharide (LPS; from *E. coli* 0111:B4; chromatographically purified), and o-phenylenediamine (OPD) were purchased from Sigma Chemical Co. (St. Louis, MO). Tween 20 was purchased from Serva (Heidelberg, Federal Republic of Germany). Purified tetanus toxin (150 Lf/ml) was kindly provided by Dr. H. G. Loggen (Rijksinstituut voor Volksgezondheid, Bilthoven, The Netherlands). It contained less than 20 pg LPS/Lf as determined with a chromogenic *Limulus* assay, which was used for all other endotoxin measurements as well (Kabivitrum, Stockholm, Sweden). Human recombinant tumor necrosis factor (hrTNF) was kindly provided by Knoll AG/BASF (Ludwigshafen, Federal Republic of Germany). The specific activity was 8×10^6 lytic units/mg protein in the 48-h L929 assay without mitomycin.

Ab. Normal human gammaglobulin (IgG) was obtained from the Central Laboratory of the Blood Transfusion Unit of the Dutch Red Cross (Amsterdam, The Netherlands). It contained less than 5 pg LPS/mg. Human antitetanus IgG (250 Lf/ml) was a gift of Dr. H. G. Loggen. It contained no detectable LPS. Goat F(ab')₂ anti-human kappa chain Ab was purchased from Tago (Burlingame, CA). It contained less than 10 pg LPS/mg. Rabbit Ab against sheep erythrocytes (SRBC) was purchased from the Rijksinstituut voor de Volksgezondheid and contained no detectable LPS. Monoclonal Ab against TNF were derived from anti-TNF secreting hybridomas obtained by a standard cell fusion procedure. Mouse spleen cells from BALB/c mice immunized with highly purified hrTNF were used for the cell fusions. Rabbit polyclonal Ab against TNF were raised by immunizing rabbits with highly purified hrTNF, and was used as unpurified serum. Peroxidase-conjugated goat anti-rabbit IgG was purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

Preparation of F(ab')₂ fragments of human IgG. F(ab')₂ fragments of human IgG were prepared by pepsin cleavage of the protein A binding fraction, which was obtained by affinity chromatography on agarose-bound protein A (Pierce, Rockford, IL). Immobilized pepsin (Pierce) was added 1:50 (w/w) to IgG in acetate buffer (pH 4.5) and allowed to cleave the Ab during 24 h at 37°C. The extent of cleavage of Ab was judged by SDS-PAGE on PhastGel System (Pharmacia Fine Chemicals, Uppsala, Sweden). Remaining intact IgG was removed from the F(ab')₂ fraction by protein A affinity chromatography. Final purity of the F(ab')₂ preparation was affirmed by SDS-PAGE.

Immune complexes. Insoluble immune complexes containing human IgG were prepared using tetanus toxin and human antitetanus IgG as described elsewhere (16). Immune complexes contained less than 125 pg LPS/mg.

Solid-phase Ab. Ab were bound to a solid phase by adding human IgG and IgG F(ab')₂ at a concentration of 100 µg/ml in phosphate buffered saline (PBS) onto 96-well immunoassay plates (Nunc, Roskilde, Denmark) for 1 h at room temperature. Plates were washed 3 times with PBS before use in the experiments.

Cells. Peripheral blood mononuclear cells (PBMC) were prepared by buoyant density centrifugation on Lymphoprep (Nycomed, Oslo, Norway) of buffy coats of donor blood kindly provided by the local Blood Bank. Monocytes were isolated from PBMC by adherence to plastic. For this purpose 5×10^6 PBMC were allowed to adhere to each well of a 24-well culture plate (Greiner, Nürtingen, Federal Republic of Germany) for 1 h at 37°C , followed by 5 washes with Hank's balanced salt solution (HBSS) to remove nonadherent cells. The adherent cell population, which will be referred to in the text as monocytes for reasons of simplicity, was shown to consist for more than 95% of alpha-naphthyl esterase positive cells. Monocytes were cultured in humidified 95% air, 5% CO_2 at 37°C in RPMI 1640 (Gibco, Paisley, Scotland) supplemented with either 10% heat-inactivated (56°C , 20 min) fetal calf serum (FCS; Boehringer, Mannheim, Federal Republic of Germany), or 2.5% heat-inactivated normal human serum (NHS; kindly provided by the local Blood Bank) together with 100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Flow, Irvine, United Kingdom). In experiments with solid-phase Ab, monocytes, obtained after adherence of 150×10^6 PBMC to 150 cm^2 culture flasks (Costar, Cambridge, MA) for 1 h at 37°C and removal of non-adherent cells by extensive washing with HBSS, were recovered by gentle scraping with a rubber policeman and subsequently added to 96-well plates coated with Ab as described before.

Phagocytosis. In phagocytosis experiments Ab-coated sheep erythrocytes (EA) were used. EA were prepared by incubating a 5% suspension of sheep erythrocytes (SRBC) in RPMI + 10% FCS with an equal volume of a sub-hemagglutinating dilution of rabbit anti-SRBC Ab in veronal buffer (pH 7.2) for 30 min at 37°C with gentle mixing. After centrifugation (3000 rpm, 6 min, 4°C) SRBC were resuspended in RPMI + 10% FCS and diluted to a final concentration of 0.1% (v/v) before addition to monocyte cultures. Unopsonized SRBC (E) at a final concentration of 0.1% (v/v) were added to monocytes as a control.

TNF-ELISA. TNF concentrations of cell-free supernatants of the cell cultures were determined with a TNF-specific enzyme-linked immunosorbent assay (ELISA). In short, immunoassay plates (96-well plates; Nunc) were coated overnight with a monoclonal anti-TNF Ab. Plates were blocked with 1% (w/v) BSA and test samples were added to the plate for 2 h at room temperature. A standard titration curve was obtained by making serial dilutions of a known sample of hrTNF. Next, the plates were washed and sequentially incubated with rabbit anti-TNF immune serum and peroxidase-conjugated goat anti-rabbit IgG, which does not cross-react with human serum proteins. After adding substrate light absorption was measured with a microelisa autoreader (Flow) using a 493 nm filter. The ELISA has a lower detection limit of 5-10 pg/ml.

RESULTS

Cross-linking of FcR on monocytes with anti-human kappa.

To examine whether cross-linking of FcR constitutes a signal for monocytes to secrete TNF, monocytes were incubated with various concentrations of goat F(ab')₂ anti-human kappa light chain and after an 8-h culture period samples of supernatant were harvested for measurement of the amount of TNF secreted. This experiment was performed both in culture medium containing 10% FCS and in culture medium containing 2.5% NHS as a source of soluble Ig. In the former circumstances the anti-human kappa associates with FcR-bound human IgG, still present on the cells as evidenced by indirect immunofluorescence using a FITC-labeled anti-human kappa Ab (data not shown), thus cross-linking monocyte FcR. In the presence of NHS the anti-human kappa will form soluble immune complexes with IgG present in the culture medium, which thereupon become cell-associated. In both culture conditions anti-human kappa induces in a dose-dependent manner TNF secretion by monocytes (Fig. 1). The TNF secretory response is shown to be about fivefold stronger when monocytes are incubated with anti-human kappa in the presence of soluble Ig in the culture

Figure 1. TNF secretion by monocytes following FcR crosslinking with an anti-human IgG Ab. Monocytes isolated as described in Methods were incubated with various concentrations of goat F(ab')₂ anti-human kappa Ab and after an 8-h culture period TNF concentration in the supernatant was determined. The left panel shows TNF concentration in the supernatant of monocytes cultured in RPMI + 10% FCS, while corresponding values obtained when monocytes were cultured in the presence of 2.5% NHS as a source of soluble Ig are shown in the right panel. Data are from one representative experiment and are expressed as mean $\pm$ s.d. of triplicate wells. Only error bars larger than symbol circumference are shown.

Figure 2. Kinetics of TNF secretion by monocytes induced by goat anti-human kappa Ab. Monocytes isolated as described in Methods were incubated with 20 μg/ml goat F(ab')₂ anti-human kappa Ab and after various time periods samples of supernatant were harvested for measurement of TNF concentration. Data are from one representative experiment and are expressed as mean $\pm$ s.d. of triplicate wells. Only error bars larger than symbol circumference are shown.

medium (Fig. 1). Next, the kinetics of the TNF secretion induced by anti-human kappa were studied. Incubating monocytes with anti-human kappa induces rapid secretion of TNF. TNF concentration in the supernatant is shown to be increased after a 2 h culture period and reaches plateau level after 12 h (Fig. 2).

Insoluble immune complexes induce TNF secretion by monocytes.

To examine whether cross-linking of FcR occurring in the course of binding and ingestion of insoluble immune complexes by monocytes is also accompanied by TNF secretion, monocytes were incubated with various amounts of immune complex, consisting of tetanus toxin and human anti-tetanus IgG. TNF concentration of the supernatant was determined after an 8 h culture period. Insoluble immune complexes induced in a dose-dependent manner TNF secretion by monocytes. The amount of immune complex optimally inducing TNF secretion was about 30 $\mu\text{g}/\text{ml}$. At higher concentrations of immune complex TNF secretion decreased (Fig. 3).

Cross-linking FcR by solid-phase Ab induces secretion of TNF by monocytes.

To exclude the possibility that TNF secretion by monocytes in the former experiments is not caused by FcR cross-linking but instead constitutes a process accompanying endocytosis of immune complexes, experiments were performed to examine

Figure 3. TNF secretion by monocytes induced by insoluble immune complexes. Monocytes isolated as described in Methods were incubated with various concentrations of insoluble immune complexes consisting of tetanus toxin and human antitetanus IgG and after an 8-h culture period TNF concentration in the supernatant was determined. Immune complexes were removed from the supernatant samples before measurement of TNF concentration in the TNF ELISA by centrifugation for 1 min at 12000 g. TNF secretion by monocytes incubated with 10 Lf/ml tetanus toxin or with 10 Lf/ml anti-tetanus IgG was equal to TNF secretion by monocytes incubated with medium alone and never exceeded 50 pg/ml. Data are from one representative experiment and are expressed as mean $\pm$ s.d. of triplicate wells. Only error bars larger than symbol circumference are shown.

Figure 4. TNF secretion by monocytes induced by solid-phase human IgG. Monocytes, isolated by plastic adherence and recovered by gentle scraping with a policeman, were added to microtiter plates coated with human IgG or IgG F(ab')₂ as described in Methods. 1×10^5 monocytes were added to each well and TNF concentration of supernatant harvested after an 8-h culture period was determined. In control wells 1×10^5 monocytes were incubated with 100 μ g/ml soluble IgG. Data from ten different donors are shown. Each open circle represents mean $\pm$ s.d. of triplicate wells of one donor. The closed square, open triangle, and closed triangle represent the mean $\pm$ s.e.m. of the ten donors combined. Only error bars larger than symbol circumference are shown.

Figure 5. TNF secretion by monocytes accompanying phagocytosis of EA. Monocytes isolated as described in Methods were incubated with 0.1% (v/v) EA. In control wells monocytes were incubated with 0.1% E or with a 1:3000 dilution of rabbit anti-SRBC Ab. TNF concentration of supernatant harvested after an 8 h culture period was determined. Data from ten different donors are shown. Each circle represents mean $\pm$ s.d. of triplicate wells of one donor. The closed square, open triangle, and closed triangle represent the mean $\pm$ s.e.m. of the ten donors combined. Only error bars larger than symbol circumference are shown.

whether FcR cross-linking by solid-phase Ab induced TNF secretion by monocytes. For this purpose monocytes, which were isolated by plastic adherence and recovered by gentle scraping with a policeman as described in Methods, were added to microtiter plates coated with normal human IgG or with IgG F(ab')₂. After an 8 h culture period samples of supernatant were harvested for determination of TNF concentration. TNF secretion by monocytes was shown to be induced by solid-phase IgG, but not by solid-phase IgG F(ab')₂ nor by soluble IgG (Fig. 4). TNF secretion was found with all donors tested, although the amount of TNF secreted varied considerably among different individuals. This further strenghtens the evidence provided by the former experiments that FcR cross-linking is the process responsible for the induction of TNF secretion by monocytes.

Phagocytosis of EA induces secretion of TNF by monocytes.

To examine whether FcR cross-linking occurring during binding and ingestion of Ab-coated particles is also accompanied by TNF secretion, monocytes were incubated with EA and after 8 h samples of supernatant were harvested for measurement of TNF concentration. EA were rapidly phagocytized by monocytes, in contrast with unopsonized SRBC. Phagocytosis of EA was accompanied by secretion of TNF, whereas incubation of monocytes with E or with rabbit anti-SRBC Ab did not lead to significant TNF secretion (Fig. 5). As for solid-phase IgG-induced TNF secretion, there also existed considerable interindividual variability in the amount of TNF secreted during phagocytosis of EA.

DISCUSSION

Several important functions of mononuclear phagocytes in immune processes are regulated through cell membrane receptors for the Fc domain of IgG (FcR). Biochemically, two different FcR have been identified on human monocytes: 1) a 72-kDa high affinity FcR (FcRI) and 2) a 40-kDa low-affinity FcR (FcRII). The FcR constitutes an important trigger molecule involved in the activation of cells of the mononuclear phagocytic system. The precise molecular mechanism underlying FcR-mediated cell activation is only partly understood. FcR on murine macrophages form ligand-dependent ion channels (20, 21) and an increase in the intracellular second messenger cAMP following FcR cross-linking has been described both in murine (22) and in human systems (23).

This study shows that FcR cross-linking induces rapid secretion of the cytokine TNF by adherent mononuclear cells. This was observed when FcR were cross-linked with soluble and insoluble immune complexes containing human IgG as well as with solid-phase human IgG. It was also found that phagocytosis of Ab-coated SRBC induced TNF secretion by monocytes. Although monocytes of all donors tested showed a TNF secretory response after FcR cross-linking, the amount of TNF secreted varied considerably among different individuals, which might be due to differences in FcR expression at the time of the experiment. In kinetic experiments in which FcR

were cross-linked with an anti-human kappa Ab TNF could already be detected in the culture supernatant after 2 h, whereas highest TNF concentration was found at 12 h. It can be excluded that TNF secretion in our experiments was in a significant way influenced by contaminating endotoxin since monocytes were shown not to produce more than 100 pg/ml TNF when incubated with amounts of endotoxin equal to those present in the experiments (data not shown). Concerning the cellular origin of TNF in our experiments, it seems most probable that it is derived from monocytes, which constituted more than 95% of the adherent cells, although we can not exclude a minor contribution by other cells like T lymphocytes and natural killer cells, which have been shown to produce TNF after stimulation (24).

TNF is a cytokine which is primarily produced by activated monocytes and macrophages but also, as has recently been shown, by other cells of hemopoietic origin (24-28). TNF was originally described as a factor, present in the serum of BCG-infected mice after injection with endotoxin, which possessed strong antitumor activity (29). Since its original description much attention has focused on the cytotoxic potential of TNF which has led to the concept of TNF as one of the primary mediators of the host antitumor response effected by activated monocytes/macrophages (30-33). The mechanism by which monocytes/macrophages respond to tumor cells with secretion of the cytotoxic TNF has however been elusive. Our finding that FcR cross-linking on monocytes induces secretion of TNF makes it conceivable that TNF is secreted in the course of ADCC against tumor cells. Similarly, TNF may be released in the course of an antibody-mediated immune response against viral and parasitic infections, which would be functional considering the antiviral (34) and antiparasitic (35) properties of TNF. Indeed, raised serum levels of TNF have been found in patients suffering from the parasitic infection kala-azar (36).

TNF, which is identical to the macrophage-derived cytokine cachectin, has been suggested to play a crucial role in the etiology of cachexia accompanying chronic infections and malignancies (37-39). Support for this hypothesis has recently emerged from a study in which cachexia was observed in mice inoculated with a TNF-producing tumor (40). Our findings provide a hypothetical model explaining the mechanism of TNF production in the course of chronic infections or malignancies. In this model chronic infection or malignancy triggers an ongoing immune response, which leads, through binding of opsonized pathogens or tumor cells to monocyte/macrophage FcR, to a continuous release of low amounts of TNF, causing metabolic derangement and cachexia.

The data also show that TNF secretion by monocytes is strongly induced by both soluble and insoluble immune complexes, which is relevant to disease states accompanied or caused by circulating or tissue-fixed immune complexes. These diseases are frequently accompanied by fever, which could be, in view of the pyrogenic properties of TNF (41), explained by immune complex-induced TNF production. Immune complex-induced TNF production could also contribute to the articular damage observed in rheumatoid arthritis. In this disease immune complexes can be detected in articular cartilage of affected joints (42). TNF has been shown to stimulate the resorption of bone and cartilage *in vitro*, partly in synergism with other cytokines, most importantly interleukin-1 (43, 44).

In summary, this study shows that the secretion of the cytotoxic and immunoregulatory cytokine TNF by monocytes can be induced through FcR cross-linking, which is relevant for immune processes in which FcR are involved, such as ADCC and phagocytosis of opsonized particles, and for disease states associated with circulating or tissue-bound immune complexes. Further study has to clarify which type of FcR is involved in the regulation of this process.

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CROSS-LINKING OF BOTH Fc γ RI AND Fc γ RII INDUCES SECRETION OF TUMOR NECROSIS FACTOR BY MONOCYTES, REQUIRING HIGH-AFFINITY Fc-Fc γ R INTERACTIONS. FUNCTIONAL ACTIVATION OF Fc γ RII BY TREATMENT WITH PROTEASES OR NEURAMINIDASE

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SUMMARY

Cross-linking of Fc γ R on human monocytes with human IgG has been shown to induce secretion of the inflammatory and immunoregulatory cytokine TNF. In the present study we examined the role of both constitutively expressed monocyte Fc γ R, the 72 kDa high-affinity Fc γ R (Fc γ RI) and the 40 kDa low-affinity receptor (Fc γ RII), in the induction of TNF secretion. On the basis of preferential binding of murine mAb of different isotype, Fc γ RI and Fc γ RII were selectively cross-linked using either solid-phase mIgG2a or solid-phase mIgG1 respectively. On freshly isolated, untreated monocytes only solid-phase mIgG2a, cross-linking Fc γ RI, induced TNF secretion. To resolve the question whether Fc γ RII was functionally incapable of inducing TNF secretion, or whether absence of induction of TNF secretion by solid-phase mIgG1 was merely due to inefficient cross-linking of Fc γ RII, the interaction between Fc γ RII and mIgG1 was enhanced by treatment of monocytes with proteases or with the desialylating enzyme neuraminidase. After treatment of monocytes with these enzymes, TNF secretion was effectively induced by solid-phase mIgG1, apparently through cross-linking of Fc γ RII. However, mIgG1-induced TNF secretion differed between protease-treated monocytes from high-responder individuals and monocytes from low-responder individuals, TNF secretion being considerably less in the latter population. To definitively prove the capacity of Fc γ RII to induce TNF secretion, experiments were performed with monocytes and mononuclear cells from individuals lacking cell membrane expression of Fc γ RI. Protease-treated monocytes and mononuclear cells from these individuals were induced to secrete TNF by solid-phase hIgG, confirming the

capacity of Fc γ RII to induce TNF secretion. It was not possible to induce TNF secretion by cross-linking Fc γ RI or Fc γ RII with anti-Fc γ R mAb and soluble or solid-phase anti-murine IgG, indicating that high-affinity Fc-Fc γ R interactions are necessary to induce cytokine release. This study demonstrates that cross-linking of both Fc γ RI and Fc γ RII induces TNF secretion by monocytes. However, Fc γ RII requires pretreatment with proteolytic enzymes or neuraminidase to become functionally active, suggesting that induction of cytokine secretion through Fc γ RII will be limited to areas of inflammation.

INTRODUCTION

Receptors for the Fc domain of immunoglobulins (FcR) play an important role in the regulation of immune processes such as phagocytosis (1,2), ADCC (3-7), clearance of immune complexes (8,9), and secretion of inflammatory mediators (10-14), and of reactive oxygen intermediates (15,16) by immune cells. On monocytes, two different FcR for IgG (Fc γ R) are constitutively expressed: a 72 kDa receptor (Fc γ RI;CD64), binding human IgG (hIgG) and murine IgG2a (mIgG2a) with high affinity ($K_a = 10^8$ - 10^9 M $^{-1}$), and a 40 kDa receptor (Fc γ RII; CD32), binding murine IgG1 (mIgG1) with low affinity ($K_a = 10^6$ M $^{-1}$) (17-20). For Fc γ RII a structural polymorphism has been demonstrated. Monocytes of approximately 30% of Caucasian individuals (low-responders, LR) fail to bind (complexed) mIgG1 to Fc γ RII, although the receptor is expressed in equal density on the cell surface compared with monocytes of the rest of the population, which bind (complexed) mIgG1 to Fc γ RII (high-responders, HR). In contrast to HR monocytes, LR monocytes therefore do not support T cell mitogenesis mediated by mIgG1 anti-CD3 mAb (21,22). Isoelectric focussing studies of Fc γ RII suggest the existence of two allelic forms of this receptor (23).

In recent years knowledge of the structure of Fc γ , both on the protein and on the gene level, has increased considerably (reviewed in 24,25). However, much remains to be learned about the function of these receptors in immune processes. On monocytes, both Fc γ RI and Fc γ RII have been shown to be capable of inducing monocyte cytotoxicity (6,7,26,27), and to mediate phagocytosis of IgG-coated particles (28). Also, Fc γ RI and Fc γ RII cross-linking has been shown to induce superoxide production by a monocyte-like cell line (16). Recently, we have shown that cross-linking of monocyte Fc γ R induces secretion of the cytotoxic and immunoregulatory cytokine tumor necrosis factor (TNF) (29). In the present study we investigated the capacity of both constitutively expressed monocyte Fc γ R to induce secretion of TNF. It is shown that cross-linking of monocyte Fc γ RI as well as of Fc γ RII induces TNF secretion, requiring high-affinity Fc-Fc γ R interactions, which is, in the case of Fc γ RII, achieved by pretreatment of monocytes with proteolytic enzymes or with neuraminidase.

MATERIALS AND METHODS

Materials. BSA (No. A-6003, essentially fatty acid free), neuraminidase (type V), pronase, phenylmethylsulfonylfluoride (PMSF), and polymyxin B sulphate were purchased from Sigma Chemical Co. (St. Louis, MO). Trypsin was purchased from Flow (Irvine, United Kingdom). Pronase and trypsin used in the binding experiments were purchased from Boehringer (Mannheim, Federal Republic of Germany). Human recombinant TNF (hrTNF), used as a standard for the TNF ELISA, was kindly provided by Knoll AG/BASF (Ludwigshafen, Federal Republic of Germany).

Antibodies. The mAb R4D10 (anti-human myosin mAb; mIgG2a), and Fab fragments of R4D10 were kindly provided by Centocor (Leiden, The Netherlands). The mAb Mac32 (anti-BSA; mIgG1) and CB1 (MOPC21; mIgG1) were kindly provided by Celltech (Slough, United Kingdom). Normal human IgG (hIgG), purified from human serum by (NH₄)₂SO₄ precipitation and DEAE-Sephadex chromatography, and consisting for more than 99.9% of IgG, was kindly provided by Dr. A. Vlug (Central Laboratory of the Netherlands Red Cross Blood Transfusion Services, CLB, Amsterdam, The Netherlands). These antibodies do not bind monocyte surface antigens with their antigen-binding site and were therefore used to cross-link monocyte FcγR by Fc-FcγR interactions. The mIgG1 anti-CD3 mAb WT31 (30) was kindly provided as unpurified ascites by Dr. W. J. M. Tax.

The following anti-FcγR mAb were used: 32.2 (16), 22 and 197 (26) (anti-FcγRI, kindly provided by Dr. M.W. Fanger, Hanover, NH); 10.1 (31) (anti-FcγRI, kindly provided by Dr. N. Hogg, London, UK); IV.3 (17) (anti-FcγRII, kindly provided by Dr. C. Anderson, Columbus, OH); CIKM5 (32,33) (anti-FcγRII, kindly provided by Dr. G. Pilkington, Melbourne, Australia).

The mAb TB-3 (IgG2a), reactive with an unknown monocyte surface antigen, was a kind gift of Dr. R.A.W. van Lier (CLB, Amsterdam, The Netherlands) (34). Goat F(ab')₂-anti-mIgG Ab (GAM) and fluorescein-conjugated goat F(ab')₂-anti-mIgG Ab were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

Solid-phase Ab. Solid-phase Ab were prepared by incubating Ab (500 μg/ml) in phosphate buffered saline (PBS, pH 7.5) overnight at 4 °C in 96-well immunoassay plates (Nunc, Roskilde, Denmark). Ab were incubated in the presence of 25 μg/ml polymyxin B sulphate, in order to eliminate any remaining endotoxin. Solid-phase mIgG1 anti-BSA were prepared by adding anti-BSA (1 mg/ml, 1 h at room temperature) to microtiter plates precoated for 1 h at room temperature with BSA (1 mg/ml in PBS). Unbound mAb were removed by three washes with PBS.

Cells. Buffy coats obtained after cytophoresis of healthy donors were kindly provided by the local Blood Bank and used to prepare monocyte cultures. In experiments with monocytes lacking cell membrane expression of FcγRI, 50 ml heparinized blood samples, obtained from three FcγRI-deficient individuals (28,35), were used to prepare cultures of peripheral blood mononuclear cells (PBMC) and monocytes. Monocytes were isolated as described elsewhere (26). In short, mononuclear cell suspensions, obtained after buoyant density centrifugation of buffy coats or heparinized blood on Lymphoprep (Nycomed, Oslo, Norway), were allowed to clump by low-speed centrifugation at 4°C. Cell clumps, consisting for more than 90-95% of monocytes, as evidenced by esterase staining, were separated from the rest of the cells by sedimentation through ice-cold bovine calf serum (BCS; Hyclone, Logan, UT). Monocytes were cultured in microtiter plates (Nunc) in humidified 95% air, 5% CO₂ at 37 °C in RPMI 1640 (Gibco, Paisley, United Kingdom) supplemented with 10% heat-inactivated BCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Flow, Irvine, United Kingdom). In each well, 5x10⁵ monocytes were cultured in 300 μl culture medium. After indicated time periods 125 μl samples of supernatant, from which cells were removed by centrifugation of the plates for 5 min at 1200 rpm, were harvested and kept frozen at -20°C for measurement of TNF concentration at a later date. In the EA-rosette assays, monocyte monolayers were prepared by adding monocytes (4x10⁵/well) to microtiter plates (Costar, Cambridge, MA) and allowing them to adhere for 45 min at 37°C, after which non-adherent cells were removed by two washes with PBS. Viability of cells, which was tested by trypan blue exclusion, exceeded 95% in all experiments.

Enzyme treatment of monocytes. Enzyme-treated monocytes were prepared by incubating monocytes at a cell density of 10x10⁶/ml in RPMI 1640 containing pronase (0.1 mg/ml), trypsin (0.5 mg/ml), or neuraminidase (1 U/ml) for 30 min at 37°C. After three washes with RPMI 1640, monocytes were

resuspended in culture medium for use in the experiments. In EA-rosette assays, monocyte monolayers were treated with enzymes at the same conditions as above. After enzyme treatment, monolayers were washed twice with PBS before use in the EA-rosette assay.

EA-rosette assay. Quantitation of binding of (rhesus D-positive) human erythrocytes (HRBC), sensitized with either hIgG (alloserum against rhesus D), or mIgG1 (anti-glycophorin A), was performed photometrically at 450 nm as described elsewhere (36). Binding indices were calculated, in order to correct for variations in background absorbance values between different experiments, as follows:

$[1 - (E_{450}/EA_{450})] \times 100 = \text{binding index}$, where E_{450} is extinction at 450 nm in wells to which HRBC without sensitizing antibody were added, and EA_{450} is extinction at 450 nm in wells to which sensitized HRBC were added. All determinations were performed in duplicate. In inhibition experiments monocyte monolayers were preincubated with the following inhibitors: mAb 10.1, which has been shown to inhibit binding of EA-hIgG to Fc γ RI (31); TB-3, which blocks monocyte Fc γ RI by a phenomenon described by Kurlander (34); IV.3 and CIKM5, which block Fc γ RII (17,32). Blocking mAb were added to monocytes in RPMI 1640 for 15 min at 4°C, after which binding of sensitized HRBC was determined. Percentage inhibition was calculated by comparing absorbances in the presence or absence of inhibitor, as described before (30).

Anti-CD3 T cell mitogenesis. To distinguish between HR and LR individuals, the capacity of the mIgG1 anti-CD3 mAb WT31 to induce T cell mitogenesis was determined. For this purpose, PBMC were cultured at a concentration of $5 \times 10^5/\text{ml}$ in round-bottomed microtiter plates (Costar), together with a $1:10^5$ dilution of WT31 ascites. After 3 days of culture, cells were pulsed for 4 h with $0.5 \mu\text{Ci}$ [^3H]thymidine ([^3H]TdR; specific activity 5 Ci/mmol; Amersham, United Kingdom), after which the cells were harvested with a cell harvester, and the radioactivity of the filters counted with a liquid scintillation counter. Proliferation was assessed in triplicate. Stimulation indexes (SI) were calculated as follows: $\text{SI} = [\text{^3H}]\text{TdR}(\text{WT31})/[\text{^3H}]\text{TdR}$, where [^3H]TdR(WT31) and [^3H]TdR represents [^3H]TdR-incorporation by PBMC incubated with or without WT31 respectively. HR were defined as having $\text{SI} > 3$.

Endotoxin measurement. Endotoxin contamination of the following antibodies was measured with a chromogenic Limulus assay (Kabivitrum, Stockholm, Sweden): R4D10: 104 pg/mg; R4D10 Fab: < 100 pg/mg; Mac32: < 100 pg/mg; hIgG: < 10 pg/mg; CB1: < 10 pg/mg; goat-anti-mIgG (Jackson): < 10 pg/mg; 32.2 (1:100 ascites): < 10 pg/ml; 22 (1:100 ascites): < 10 pg/ml; 197 (1:10 hybridoma supernatant): 73 pg/ml; IV.3 (1:10 hybridoma supernatant): 45 pg/ml. Since the enzymes neuraminidase, pronase, and trypsin interfered with the Limulus assay, endotoxin contamination of these enzyme preparations could not be determined. Endotoxin contamination of RPMI 1640, BCS, and Lymphoprep were controlled regularly by Limulus assay, and never exceeded 50 pg/ml.

TNF ELISA. TNF concentration of the supernatants was determined with a TNF-specific ELISA (37), with slight modifications as described before (29).

Statistics. Student's t-test for paired and unpaired data was used for statistical analysis of the data. Differences were considered significant at the $p < .05$ level.

RESULTS

Solid-phase mIgG2a, but not solid-phase mIgG1 induces TNF secretion by monocytes.

We used solid-phase mIgG2a or mIgG1 to selectively cross-link either Fc γ RI or Fc γ RII on monocytes. Solid-phase mIgG2a, but not mIgG1, induced secretion of TNF by freshly isolated monocytes (Fig. 1), suggesting that cross-linking of Fc γ RI, but not of Fc γ RII, triggers secretion of TNF by monocytes. Fc-dependency of mIgG2a-induced TNF secretion was demonstrated by the absence of TNF secretion by monocytes incubated with solid-phase mIgG2a Fab (Fig. 1).

Figure 1. Solid-phase mIgG2a, but not mIgG1, induces secretion of TNF by monocytes. Monocytes were incubated for 18 h with either fluid-phase or solid-phase mIgG1 (Mac32), mIgG2a (R4D10), or mIgG2a Fab (R4D10 Fab), after which samples of supernatant were harvested for measurement of TNF concentration. Solid-phase mAb were prepared as described in Methods. Fluid-phase mAb were added to monocytes at concentrations equal to those used to prepare solid-phase mAb, e.g. 1 mg/ml (Mac32 and R4D10) or 500 μ g/ml (R4D10 Fab). Data represent mean $\pm$ s.e.m. of triplicate determinations of 10 (mIgG1), 14 (mIgG2a), or 8 (mIgG2a Fab) individuals.

* $p < .001$.

Figure 2. Treatment of monocytes with pronase or neuraminidase enhances binding of EA-mIgG1. Monocyte monolayers were treated with pronase or neuraminidase as described in Methods. Next, binding of HRBC, sensitized with mIgG1 anti-glycophorin A ascites at three different dilutions, was determined as described in Methods. Data represent the mean of duplicate determinations of one of four different experiments with similar results.

Figure 3. Inhibition of binding of EA-mIgG1 or EA-hIgG to neuraminidase-treated monocytes by mAb blocking either FcγRI or FcγRII. Monocyte monolayers were treated with neuraminidase as described in Methods. After neuraminidase treatment monolayers were washed once with PBS, incubated with culture medium for 15 min, again washed once with PBS, after which mAb blocking either FcγRI (10.1 1:250 ascites, TB-3 1:100,000 ascites) or FcγRII (IV-3 1:20 hybridoma culture supernatant, CIKM5 1:50 purified IgG) were added to the monocytes for 15 min. Next, binding of EA-mIgG1 or EA-hIgG was determined as described in Methods. Data are expressed as percentage inhibition of EA-rosetting as defined in Methods, and represent the mean of duplicate determinations of one of three different experiments with similar results.

Figure 4. Solid-phase mIgG1 induces TNF secretion by monocytes after enzyme treatment. Monocytes were treated with neuraminidase (1 U), pronase (0.1 mg/ml) or trypsin (0.5 mg/ml) as described in Methods. After three washes with culture medium monocytes were added to microtiter plates coated with mIgG1 (CB1, 1 mg/ml; solid bars). Enzyme-treated monocytes added to uncoated wells served as controls (open bars). After 18 h samples of supernatant were harvested to determine TNF concentration. Data represent mean $\pm$ s.e.m. of triplicate determinations of 6 different individuals.

* $p < .001$ vs. control.

** $p < .01$ vs. control.

Treatment of monocytes with neuraminidase or pronase increases the interaction between mIgG1 and Fc γ RII.

In a recent study by one of us it was shown that the interaction between Fc γ RII and mIgG1 increases after treatment of monocytes with proteolytic enzymes (38). In this study it was also demonstrated that treatment of monocytes with proteolytic enzymes increases the affinity of Fc γ RII for hIgG. Since Fc γ R are sialoglycoproteins we were interested to know whether treatment of monocytes with the desialylating enzyme neuraminidase also affects the interaction between Fc γ R and mIgG1. Treatment of monocytes with either the proteolytic enzyme pronase or with neuraminidase strongly enhanced binding of human erythrocytes sensitized with mIgG1 (EA-mIgG1) (Fig. 2). Control experiments were performed to exclude the possibility that the observed increase in binding of EA-mIgG1 after neuraminidase treatment was attributable to proteolytic enzymes contaminating the neuraminidase preparation used. The increase in binding of EA-mIgG1 after neuraminidase treatment was not affected by pretreatment of neuraminidase with the protease-inhibiting compound PMSF (0.5 mM, 30 min; 0% reduction in binding vs. cells treated with neuraminidase in the absence of PMSF; data from 4 different individuals). The increase in binding of EA-mIgG1 after pronase treatment was however largely abolished by pretreatment of pronase with PMSF (90.7 $\pm$ 8.1 reduction in binding vs. cells treated with pronase not pretreated with PMSF; mean $\pm$ s.e.m. of four different individuals). The increase in binding was consistently found to be greater after treatment of monocytes with pronase than after neuraminidase treatment. Increased binding of EA-mIgG1 to neuraminidase-treated monocytes could, as has been shown for proteolytic enzyme-treated monocytes in the above-mentioned study (38), be inhibited almost totally by the Fc γ RII-blocking mAb IV.3 and CIKM5, but not or only minimally by the Fc γ RI-blocking mAb 10.1 and TB-3. In contrast, rosetting between neuraminidase-treated monocytes and EA-hIgG was strongly inhibited by mAb 10.1 and TB-3, but only minimally by mAb IV.3 and CIKM5, indicating that after neuraminidase treatment rosetting between monocytes and EA-hIgG is predominantly mediated by Fc γ RI (Fig. 3). These data indicate that increased binding of mIgG1 to monocytes after treatment with proteolytic enzymes or neuraminidase can be accounted for by an increased interaction between Fc γ RII and mIgG1.

Solid-phase mIgG1 induces TNF secretion by monocytes treated with proteolytic enzymes or neuraminidase.

Since the interaction between Fc γ RII and mIgG1 increases considerably after treatment of monocytes with proteolytic enzymes or neuraminidase, we investigated whether incubation of enzyme-treated monocytes with solid-phase mIgG1 leads to TNF secretion. Solid-phase mIgG1 significantly increased TNF secretion above control values after enzyme treatment (Fig. 4). The increase in TNF secretion was greater after treatment of monocytes with proteolytic enzymes than with neuraminidase, paralleling the greater increase in interaction between Fc γ RII and mIgG1 on pronase-treated monocytes observed in the rosetting experiments. These data indicate that cross-linking

Figure 5. Solid-phase mIgG1-induced TNF secretion by HR or LR monocytes after protease treatment. Monocytes from four HR individuals (squares) or four LR individuals (circles) were treated with pronase (0.1 mg/ml) as described in Methods. After three washes with culture medium monocytes were added to microtiter plates coated with mIgG1 (CB1, 1 mg/ml). Untreated monocytes incubated with solid-phase mIgG1 served as controls. After 18 h samples of supernatant were harvested to measure TNF concentration. Data represent the means of triplicate determinations. TNF secretion by monocytes incubated in uncoated wells was always less than 200 pg/ml in these experiments.

* $p < .01$ vs. control.

**not significant vs. control.

of Fc γ RII leads to TNF secretion only after the interaction between this receptor and the cross-linking mIgG1 antibody has been increased above a certain threshold by pretreatment of the monocytes with proteolytic enzymes or neuraminidase.

Difference in solid-phase mIgG1-induced TNF secretory response between protease-treated monocytes from HR or LR individuals.

Since the affinity of Fc γ RII for mIgG1 is markedly different between monocytes from HR or LR individuals, we examined the influence of HR or LR phenotype on the solid-phase mIgG1-induced TNF secretion by protease-treated monocytes. Solid-phase mIgG1-induced TNF secretion by HR monocytes was significantly increased after protease treatment. No significant increase in TNF secretion was found with monocytes from four LR individuals (Fig. 5). This finding supports the contention that effective induction of TNF secretion is dependent on strong interactions between Fc γ RII and the cross-linking mIgG1 mAb.

Protease-treated monocytes from Fc γ RI-deficient individuals secrete TNF after incubation with solid-phase hIgG.

Hypothetically, it remained possible that, through increased interaction between Fc γ RI and mIgG1 after enzyme treatment of monocytes, TNF secretion was induced by cross-linking of Fc γ RI rather than Fc γ RII. To definitively prove the capacity of Fc γ RII

Figure 6. TNF secretion by monocytes or PBMC from Fc γ RI-deficient individuals after treatment with proteolytic enzymes. Monocytes from three Fc γ RI-deficient individuals were treated with proteolytic enzymes as described in Methods. Enzyme-treated or control monocytes (5×10^4 cells/well) were incubated with solid-phase hIgG for 18 h, after which samples of supernatant were harvested for measurement of TNF concentration (Fig. 6A). Each bar represents the mean of duplicate determinations of one experiment. TNF secretion by enzyme-treated monocytes from all three individuals in the absence of solid-phase hIgG was below the detection limit (e.g. <10 pg/ml). The same experiment was performed using pronase-treated PBMC (2.5×10^5 cells/well; Fig. 6B). TNF secretion by pronase-treated PBMC in the absence of solid-phase hIgG varied between 60-70 pg/ml for all three individuals.

to induce TNF secretion, we performed experiments with cells from individuals who have an inherited defect in cell membrane expression of Fc γ RI (28,35). Since only Fc γ RII is expressed on these cells, solid-phase hIgG could be used instead of solid-phase mIgG1 to cross-link this receptor. The affinity of Fc γ RII for hIgG has been shown to increase after proteolysis (38). Protease-treated monocytes from three different Fc γ RI-deficient individuals were shown to secrete TNF above control levels when incubated with solid-phase hIgG (Fig. 6A). Similar data were obtained when the experiment was performed with unseparated PBMC from these individuals (Fig. 6B). In both experiments the highest increase in TNF secretion was observed with cells from donor 2. This donor was of HR phenotype, whereas donors 1 and 3 were of LR phenotype, which likely explains the difference in TNF secretion between these donors. These data substantiate the capacity of Fc γ RII to induce TNF secretion after its affinity

Figure 7. Cross-linking of Fc γ R with different anti-Fc γ R mAb does not induce TNF secretion by monocytes. Monocytes were incubated with anti-Fc γ RI mAb 32.2, 22, and 197, or with the anti-Fc γ RII mAb IV.3 for 30 min at 4 °C. Concentrations of anti-Fc γ R mAb giving optimal binding, as determined by indirect immunofluorescence, were used to coat monocytes. After three washes with culture medium monocytes were incubated for 18 h with different concentrations of GAM to cross-link Fc γ R, after which samples of supernatant were harvested to determine TNF concentration. Data represent mean of triplicate determinations of four different individuals. Error bars have been omitted for clarity.

for the Fc domain of the cross-linking Ab has been increased by enzyme treatment. Moreover, these data also demonstrate that Fc γ RII can be effectively cross-linked by hIgG after protease treatment, suggesting that this process is relevant for in vivo inflammatory processes.

Cross-linking of Fc γ RI or Fc γ RII with anti-Fc γ R mAb does not lead to TNF secretion.

The former experiments suggest that effective induction of TNF secretion through cross-linking of either Fc γ RI or Fc γ RII depends upon high-affinity interactions of Ab with Fc γ R. To examine this hypothesis we used monoclonal anti-Fc γ R Ab, which bind with high affinity to Fc γ R, to cross-link both Fc γ RI and Fc γ RII. These mAb cross-link Fc γ R not by Fc-Fc γ R interactions, but by binding with their antigen-binding site to different epitopes on the Fc γ R. Monocytes were incubated with optimal concentrations of one of the following mAb: 32.2, 22, or 197 (anti-Fc γ RI mAb), and IV.3 (anti-Fc γ RII mAb). Concentrations of anti-Fc γ R mAb giving optimal binding were determined by indirect immunofluorescence on monocytes as evaluated by FACS analysis. Fc γ R were cross-linked by adding soluble GAM at concentrations ranging between 0.1-100 $\mu\text{g/ml}$ to monocytes coated with the different anti-Fc γ R mAb. Efficacy of cross-linking was judged visually by fluorescence microscopy of monocytes incubated at 37°C with anti-Fc γ R mAb and fluorescein-conjugated GAM in the absence of sodium azide. Capping of Ab, indicating effective Fc γ R cross-linking, was observed at concentrations

of GAM between 10-30 $\mu\text{g/ml}$. Monocytes did not secrete TNF above control values when incubated with any of the anti-Fc γ R mAb and GAM at concentrations between 1-30 $\mu\text{g/ml}$ (Fig. 7). Neither was TNF secretion observed when the cross-linking GAM was bound to a solid-phase (data not shown). These findings strongly suggest that high-affinity Fc-Fc γ R interactions are necessary to induce TNF secretion by monocytes upon Fc γ R cross-linking.

DISCUSSION

In this study we investigated the induction of secretion of the immunoregulatory and inflammatory cytokine TNF by cross-linking of both constitutively expressed Fc γ R, Fc γ RI and Fc γ RII, on human peripheral blood monocytes. On untreated monocytes, TNF secretion was induced by solid-phase mIgG2a, cross-linking Fc γ RI, but not by solid-phase mIgG1, cross-linking Fc γ RII. Since it has been shown that immobilized mIgG1 immune complexes effectively modulate Fc γ RII from the apical surface of adherent monocyte cultures (39), these findings at first sight suggest that TNF secretion can only be induced by cross-linking of Fc γ RI. However, after treatment of monocytes with neuraminidase or proteolytic enzymes, TNF secretion could also be induced by solid-phase mIgG1, cross-linking Fc γ RII. This is not a result of enzyme treatment itself, since 'spontaneous' TNF secretion by enzyme-treated monocytes (probably a result of stimulation of monocytes by endotoxin contaminating the enzyme preparations used), cultured in medium alone (Fig. 4), or in the presence of fluid-phase mIgG1 (data not shown), was significantly lower than TNF secretion by enzyme-treated monocytes incubated with solid-phase mIgG1.

The rosetting experiments show that treatment of monocytes with neuraminidase increases the interaction between Fc γ RII and mIgG1. The same has recently been demonstrated after treatment of monocytes with proteolytic enzymes (38). Moreover, in the same study an increased binding affinity of Fc γ RII for hIgG after protease treatment has also been demonstrated. Others have demonstrated increased binding of rabbit IgG immune complexes to monocyte Fc γ R after treatment with proteolytic enzymes (40,41). At the time of these studies, Fc γ RII had not yet been discovered. Their findings can however be readily explained by an increased affinity of Fc γ RII for rabbit IgG. In summary, increased interaction between Fc γ RII and mIgG1 is found after treatment with both neuraminidase and proteases, whereas the binding affinity of Fc γ RII for hIgG (and possibly also rabbit IgG) is increased after proteolysis.

Increased interaction between Fc γ RII and mIgG1, enhancing the strength of Fc γ RII cross-linking, constitutes a likely explanation for solid-phase mIgG1-induced TNF secretion by monocytes treated with neuraminidase or with proteases. The contention that induction of TNF secretion by Fc γ RII cross-linking is dependent on strong interactions between Fc γ RII and the cross-linking Ab is supported by the finding that solid-phase mIgG1 enhances TNF secretion by pronase-treated monocytes from HR individuals, but not or only minimally by monocytes from LR individuals. Since the difference in affinity of Fc γ RII of HR or LR individuals is a relative rather than an absolute one, it remains possible that the inability to induce TNF secretion by LR

monocytes in these experiments is caused by insufficient cross-linking by the aspecifically coated mIgG1 mAb. This conclusion is supported by the finding that, when using solid-phase hIgG instead of solid-phase mIgG1 to cross-link Fc γ RII in the experiments with Fc γ RI-deficient cells described in figure 6, LR cells are also induced to secrete TNF, although to a lesser extent than HR cells. Apparently, Fc γ RI are cross-linked more strongly by solid-phase hIgG than by solid-phase mIgG1. Furthermore, these experiments, performed with cells expressing only Fc γ RI on their cell surface, convincingly demonstrate the ability of Fc γ RII to induce TNF secretion.

It was not possible to induce TNF secretion by cross-linking Fc γ RI or Fc γ RII with anti-Fc γ R mAb, which bind with their antigen-binding site to various epitopes on Fc γ R, together with fluid-phase GAM. Our previous study showed that TNF secretion can be induced effectively by cross-linking cytophilic hIgG with a fluid-phase anti-hIgG Ab (29). The inability to induce TNF secretion with anti-Fc γ R mAb and fluid-phase GAM seems therefore not to be due to Fc γ R being less effectively cross-linked with fluid-phase Ab as compared to solid-phase Ab. Moreover, TNF secretion was neither induced by anti-Fc γ R mAb and solid-phase GAM. We therefore conclude that high-affinity Fc-Fc γ R interactions are required to trigger secretion of the cytokine TNF by monocytes. These findings are in contrast with those of others, who were able to induce superoxide production by a human monocyte-like cell line by cross-linking Fc γ RI and Fc γ RII with anti-Fc γ R mAb as well as with hIgG-immune complexes (16). It has also been shown that both Fc γ RI and Fc γ RII are cytotoxic trigger molecules on monocytes, when using hybridoma cells expressing mAb 32.2 or IV.3 on their cell surface as target cells (6,7,26). Furthermore, an increase in intracellular Ca²⁺-levels has recently been observed in the monocyte cell line U937 after cross-linking Fc γ RI and Fc γ RII with anti-Fc γ R mAb as well as via Fc-Fc γ R interactions (41). Our study strongly suggests that, for as yet unknown reasons, effective induction of TNF secretion requires binding of the Fc moiety of the cross-linking Ab to the Fc γ R.

TNF is a cytotoxic and immunoregulatory cytokine with a broad range of activities on different cell types (reviewed in 43). Generally speaking, it can be viewed as a cytokine which enhances immune and inflammatory processes. It also has strong pyrogenic activity and induces the production of other pyrogens, like interleukin-1 (44), and interleukin-6 (45). The findings of the present study suggest that circulating monocytes will only be able to secrete TNF via Fc γ RI cross-linking, occurring for instance by circulating immune complexes or antibody-coated pathogens (29). Since the affinity of Fc γ RI for complexed IgG or monomeric IgG does not differ much (46), it can be expected that Fc γ RI cross-linking is strongly inhibited by the excess of monomeric IgG present in the circulation, thereby protecting the organism against massive release of a potentially lethal cytokine. In areas of inflammation, where proteases, released by granulocytes, and neuraminidase, derived for instance from bacteria or viruses, are likely to be present, Fc γ RII will be 'activated', becoming functional with respect to the induction of TNF secretion. This provides a mechanism limiting cytokine release to areas of inflammation.

It is known from other studies that the expression and function of Fc γ R can be modulated by treatment with proteolytic enzymes. The high-affinity Fc γ R on mouse macrophages loses its capacity to bind monomeric mIgG2a after treatment with try-

psin, whereas the low-affinity Fc γ R present on these cells retains its capacity to bind polymeric mIgG1/mIgG2b (47). Recently, the protease elastase was shown to differentially modulate the expression and function of both Fc γ R constitutively present on human neutrophils (48). Our study shows that not only proteolytic enzymes but also the deglycosylating enzyme neuraminidase is capable of influencing Fc γ R function. Interestingly, neuraminidase has been shown to influence other immune responses as well. For example, MHC-restricted alloreactive T cell activation by different cells can be enhanced by pretreatment of these cells with neuraminidase (49-51). Although the significance of these findings remains to be learned, modulation of immune responses or uncovering of latent immune responses by enzymes released in areas of inflammation may constitute an important host mechanism to fine-tune immune processes in areas of active inflammation.

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THE INHIBITORY EFFECT OF CORTICOSTEROIDS ON THE SECRETION OF TUMOR NECROSIS FACTOR BY MONOCYTES, INDUCED BY DIFFERENT STIMULATORY AGENTS

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SUMMARY

The cytokine tumor necrosis factor is believed to be involved in the pathophysiology of several human disease states, both septic and non-septic. Different pathways of induction are involved in the generation of TNF in these disease states. We therefore used four different stimulatory agents, i.e. LPS, PMA, silica quartz, and anti-human IgG Ab, to study the influence of the corticosteroids prednisolone and budesonide on the secretion of TNF by human monocytes. Both prednisolone and budesonide inhibited TNF secretion induced by these four stimulating agents in a different degree. Inhibition was strong when TNF secretion was induced by LPS or anti-human IgG Ab. A weaker inhibitory effect was observed when TNF secretion was induced by silica quartz. Only minimal inhibition of PMA-induced TNF secretion was observed. Furthermore, it is shown that inhibition is dependent on the dose of corticosteroid, but not or only minimally on the dose of stimulating agent, indicating that inhibition cannot be overcome by increasing the cell-activating stimulus. Finally, optimal inhibition of TNF secretion by corticosteroids is shown to be dependent on the presence of corticosteroids during the phase of cell stimulation.

INTRODUCTION

Tumor necrosis factor (TNF) is an inflammatory and immunoregulatory cytokine produced mainly by monocytes and macrophages after stimulation with endotoxin (1,2), but also by other cells of the immune system like T lymphocytes (3) and natural killer cells (4). TNF has numerous regulatory functions in immune processes, thus playing a pivotal role in the enhancement of inflammation and immunity (5). Besides its physiological role TNF is also believed to be involved in the pathophysiology of a

number of disease processes like sepsis (6,7), cachexia or anorexia accompanying cancer or chronic infection (8-10), parasitic infections (11,12), allograft rejection (13), graft-vs-host disease (14), and autoimmune diseases like rheumatoid arthritis (15-17) and SLE (18).

The corticosteroid drugs prednisolone and budesonide have both been shown to inhibit inflammatory processes and to suppress the immune response in several autoimmune diseases or during allograft rejection (19,20). One of the important mechanisms underlying the antiinflammatory and immunosuppressive effects of corticosteroids is the inhibition of production and secretion of inflammatory mediators by immunocompetent cells (19,21). For example, corticosteroids have been shown to inhibit production of the interleukins IL-1 and IL-2, and of interferon- γ (22-24).

Corticosteroids have also been shown to inhibit TNF production by murine and rat macrophages (1,25) as well as by human monocytes (26). In these studies endotoxin was used to stimulate TNF production. Besides endotoxin, other compounds have been shown to induce TNF production. The protein kinase C-activating agent PMA is a potent inducer of TNF production by monocytes (27). Recently, we have demonstrated that TNF secretion by monocytes can be induced through cross-linking of monocyte Fc γ receptors (Fc γ R), for example with an anti-human IgG Ab or with immune complexes (28). Furthermore, data from our laboratory demonstrate that silica quartz, which is a known inducer of interleukin 1 (IL-1) production in monocytes (29), can also induce TNF production by monocytes (30). We therefore investigated the influence of corticosteroids on the secretion of TNF by monocytes after stimulation with these different compounds.

It is shown that TNF secretion is variably inhibited by corticosteroids, depending on the type of stimulus used. The strongest inhibition was found when TNF secretion was induced by LPS or anti-human IgG Ab, whereas inhibition of PMA- and silica-induced TNF secretion was considerably weaker.

MATERIALS AND METHODS

Materials. Prednisolone was purchased from Centrachemie (Etten-Leur, The Netherlands). A stock solution was prepared of 25 mg/ml prednisolone in water. Budesonide was kindly provided by Dr. R. Brattsand (Drako/Astra, Lund, Sweden). A stock solution of 10^{-2} M budesonide was prepared in 96% ethanol. Both stock solutions were further diluted in culture medium to obtain the final concentrations used in the experiments. Monocytes cultured in medium containing equivalent amounts of ethanol without budesonide were used as controls for budesonide-treated cells. Monocytes cultured in medium without any additions were used as controls for the prednisolone-treated cells. Lipopolysaccharide (LPS; from *E. coli* 055:B5) was purchased from Difco (Detroit, MI, USA). 4- β -phorbol-12-myristate-13-acetate (PMA) and silica quartz (particle size 0.007 μ m) were purchased from Sigma (St. Louis, MO, USA). Goat F(ab') $_2$ anti-human kappa chain Ab was purchased from Tago (Burlingame, CA, USA).

Cells. Buffy coats obtained after cytophoresis of healthy donors were kindly provided by the local Blood Bank. Monocytes were isolated as described elsewhere (31). In short, mononuclear cell suspensions obtained after Ficoll-Isopaque centrifugation were allowed to clump by low speed centrifugation at 4 °C. Cell clumps, consisting for more than 90-95% of monocytes as evidenced by esterase staining, were separated from the rest of the cells by sedimentation through ice-cold bovine calf serum (BCS; Hyclone, Logan, UT, USA). Monocytes were cultured in 96-well flat-bottom culture plates (Costar, Cambridge, MA, USA) in humidified 95% air, 5% CO $_2$ at 37 °C in RPMI 1640 (Gibco, Paisley, UK) supplemented

with 10% heat-inactivated BCS, 100 IU/ml penicillin and 100 μ g/ml streptomycin (Flow, Irvine, UK). In each well 2.5×10^4 monocytes were cultured in 300 μ l culture medium. After indicated time periods 125 μ l samples of supernatant, from which cells were removed by centrifugation of the plates for 5 min at 1200 rpm, were harvested and kept frozen at -20°C for measurement of TNF concentration at a later date. Viability of cells, which was determined by trypan blue exclusion, exceeded 95% in all experiments. All experiments were performed in duplicate or triplicate.

TNF ELISA. TNF concentration of the supernatants was determined with a TNF-specific ELISA (32), with slight modifications as described before (28).

Statistics. Student's t-test for paired and unpaired data was used for statistical analysis of the data. Differences were considered significant at the $p < .05$ level.

RESULTS

LPS, PMA, silica, and anti-human kappa chain Ab were used as stimulating agents to study the influence of corticosteroids on TNF secretion by monocytes. Concentrations of each of the stimulating agents giving optimal stimulation of TNF secretion were determined in separate experiments (data not shown). These concentrations were used in the first set of experiments, in which the effect of a four hour preincubation period with various concentrations of the corticosteroids prednisolone and budesonide on TNF secretion by monocytes was studied (Fig. 1). TNF secretion was determined by measuring TNF concentration of the cell-free supernatant of monocyte cultures incubated for 17 h with the stimulating agents in the presence or absence of corticosteroids. It is shown that both prednisolone and budesonide inhibit TNF secretion in a dose-dependent manner. The strongest inhibition was found when TNF secretion was induced by LPS or anti-hIgG Ab. PMA-induced TNF secretion was only moderately inhibited, especially with prednisolone, which even enhanced TNF secretion at lower concentrations. Silica-induced TNF secretion was also inhibited by both corticosteroids, the degree of inhibition being less than that observed after stimulation of TNF secretion by LPS or anti-hIgG Ab.

Next, we examined whether the degree of inhibition of TNF secretion by corticosteroids was dependent on the dose of stimulating agent added to the monocytes. As an example of the dose-response relationships of LPS-induced TNF secretion, data from two individuals are given in figure 2, panel A. Two different concentrations of the corticosteroids prednisolone and budesonide were used to inhibit TNF secretion, one giving optimal inhibition of TNF secretion and one giving suboptimal inhibition of TNF secretion, as determined by the experiments shown in fig. 1. At optimal inhibitory concentrations of prednisolone and budesonide inhibition of TNF secretion was not affected by the dose of LPS added to the monocyte cultures. However, at suboptimal inhibitory concentrations of both corticosteroids a distinct reduction in inhibition was observed when the amount of LPS added to the cultures was increased (Fig. 2, panels B and C). The same was essentially found when TNF secretion was induced through cross-linking of monocyte Fc γ R with anti-hIgG Ab. The influence of the amount of anti-hIgG Ab on the degree of inhibition by corticosteroids was however less than that observed with LPS (Fig. 3).

Next, we examined whether the inhibition of TNF secretion by corticosteroids requires the presence of corticosteroids at the time of addition of stimulating agents to

Figure 1. Inhibition by corticosteroids of TNF secretion induced by four different stimulating agents. Monocytes were incubated with various concentrations of either prednisolone (panel A) or budesonide (panel B). After four hr the stimulating agents were added to the cultures. After 17 hr, samples of supernatant were harvested to measure TNF concentration. Data represent mean $\pm$ s.e.m. of triplicate determinations of four different individuals. TNF secretion is expressed as percentage of TNF secretion in the absence of corticosteroids.

the monocytes. Monocytes were preincubated for 4 hours with prednisolone or budesonide at optimal inhibitory concentrations, after which the corticosteroids were washed away. Four hours later monocytes were stimulated with LPS. As shown in table I, monocyte TNF secretion was not inhibited by a four hour preincubation period with prednisolone. TNF secretion was even enhanced by preincubation with prednisolone, although the difference did not reach statistical significance. After a four hour preincubation period with budesonide, TNF secretion was still significantly suppressed (Table I). The degree of inhibition of TNF secretion was however significantly less than that observed in the continual presence of budesonide ($25 \pm 11\%$ inhibition vs. $80 \pm 14\%$ inhibition, $m \pm \text{s.e.m.}$ of four experiments; $p < .01$), indicating that the inhibition of TNF secretion is partially reversed after removal of budesonide from the cultures.

DISCUSSION

In this study it is shown that corticosteroids inhibit the secretion of TNF by human monocytes in a dose-dependent manner. Both prednisolone, which is used as a systemic immunosuppressive drug in clinical medicine, and budesonide, which is used mainly as a topical antiinflammatory drug in respiratory diseases, were shown to inhibit TNF secretion. Budesonide proved to be a more potent inhibitor of TNF secretion than prednisolone (approximately 100 times more potent on a molar basis). Corticosteroids variably inhibited TNF secretion, depending on the type of stimulus used to induce TNF secretion. The strongest inhibition was observed when TNF secretion was induced by a 'receptor-mediated' mechanism, i.e. by LPS or through cross-linking of monocyte $\text{Fc}\gamma$ receptors with an anti-hIgG Ab. The inhibition was much less or, at lower concentrations of corticosteroids, even totally absent, when TNF secretion was induced by the protein kinase C-activating agent PMA. The inhibition observed when TNF secretion was induced by silica, which non-specifically activates monocytes, was intermediate in comparison with PMA-induced and LPS/anti-hIgG-induced TNF secretion. These data suggest that, as far as cytokine secretion is concerned, corticosteroids interfere more potently with receptor-transmitted cellular activating pathways than with protein kinase C-mediated cellular activation.

The results shown in figures 2 and 3 indicate that the inhibition by corticosteroids of TNF secretion induced by LPS or anti-hIgG Ab is not of a competitive nature. The inhibition by optimal inhibitory concentrations of corticosteroids cannot be eliminated by increasing the dose of stimulatory agents. Only at suboptimal inhibitory concentrations of corticosteroids a slight reduction of inhibition was observed when the concentration of LPS or anti-hIgG Ab was increased.

Prednisolone-induced inhibition was shown to be rapidly reversible. Four hours after removal of prednisolone from the culture medium inhibition of TNF secretion was no longer observed. Budesonide-induced inhibition was less rapidly reversed, significant inhibition still being present four hours after removal of budesonide from the cultures. Since the washing procedure was identical for both corticosteroids, it seems unlikely that the inhibition observed after preincubation with budesonide is due

Figure 2. Influence of the concentration of LPS on inhibition of TNF secretion by corticosteroids. Monocytes were preincubated with budesonide (panel B) or prednisolone (panel C) at concentrations giving optimal (open bars) or suboptimal (solid bars) inhibition of LPS-induced TNF secretion, as determined by the former experiments. Optimal inhibitory concentrations chosen were 10^{-7} M and 2×10^{-5} M for budesonide and prednisolone respectively. Suboptimal inhibitory concentrations chosen were 10^{-9} M and 2×10^{-7} M for budesonide and prednisolone respectively. Four hr later LPS at different concentrations was added to the cultures. After 17 hr samples of supernatant were harvested to measure TNF concentration. Values in panel B and C are means of triplicate determinations of two different individuals. Data are expressed as percentage inhibition of TNF secretion, which is defined as $[1 - (\text{TNF with corticosteroids}) / (\text{TNF without corticosteroids})] \times 100$. Panel A shows the actual dose-response curves of LPS-induced TNF secretion in these two individuals (expressed as mean of triplicate determinations of both individuals).

to incomplete removal of the drug from the culture medium, since this would have resulted in persistent inhibition in the prednisolone-pretreated cultures as well. The observed difference in duration of inhibition suggests that budesonide is less rapidly cleared from the intracellular compartment, possibly because of greater affinity for intracellular steroid-binding receptor molecules. This is also in accordance with the finding that budesonide is, on a molar basis, a far more potent inhibitor of TNF secretion than prednisolone.

Endotoxin-induced TNF production has been shown to be inhibited by corticosteroids in previous studies, both in murine and in human systems (1,25,26). Dexamethasone was shown to inhibit endotoxin-induced mouse macrophage TNF production at transcriptional as well as posttranscriptional level (1). Only short periods of exposure to dexamethasone were necessary to fully block TNF production, inhibition even being present when dexamethasone was administered to the cells at the same time as endotoxin. Human monocyte TNF production induced by endotoxin was also shown to be inhibited by dexamethasone, requiring however a much longer period (48 h) of preincubation of cells with dexamethasone for inhibition to become optimal (26). Our study shows that corticosteroids also inhibit monocyte TNF secretion induced by stimuli other than endotoxin. We also found that only short periods of exposure (4 h) to prednisolone or budesonide were needed to optimally inhibit TNF secretion.

TNF is believed to be one of the prime mediators of sepsis, on the basis of both experimental animal studies (6,33) and clinical studies in man (7,34,35). Numerous animal studies have demonstrated a protective effect of corticosteroids against mortality in experimental sepsis (reviewed in 36). However, a beneficial effect of corticosteroids in human sepsis has not yet been convincingly demonstrated (36-38). The strong inhibitory effect of corticosteroids on endotoxin-induced TNF production may be one of the mechanisms underlying the protective effect of corticosteroids in experimental animal sepsis.

We have recently demonstrated that TNF secretion by monocytes can be induced through cross-linking of the Fc γ -receptor, for example with immune complexes or with an anti-hIgG Ab (28). This finding suggested to us that TNF may play a role in the pathophysiology of immune complex diseases, for example rheumatoid arthritis. Several investigations support such a hypothesis. TNF has been shown to induce bone and cartilage resorption *in vitro*, partly in synergism with interleukin 1 (IL-1) (15, 16,39). Furthermore, TNF has recently been detected in synovial fluid of patients with rheumatoid arthritis (40). These disease states can be potentially suppressed by corticosteroids. Our finding that corticosteroids inhibit TNF secretion induced through Fc γ R cross-linking contributes to the understanding of the mechanisms underlying the beneficial effect of corticosteroids in these disease states.

TNF shares many of its biological activities with another important immunoregulatory cytokine, IL-1. Corticosteroids have also been shown to inhibit IL-1 synthesis by human monocytes (22) and by a human monocyte-like cell line (23). IL-1 synthesis by monocytes is mainly inhibited at post-transcriptional level (22). The exact cellular level at which corticosteroids interfere with human monocyte TNF production remains subject of further study.

Figure 3. Influence of the concentration of anti-hIgG Ab on inhibition of TNF secretion by corticosteroids. Legend is the same as for Fig. 2, with anti-human IgG Ab being the stimulating agent instead of LPS.

TABLE I. Effect of preincubation of monocytes with corticosteroids on LPS-induced TNF secretion.^a

preincubation with:	LPS added (ng/ml)	TNF (pg/ml)
-	100	4458 ± 2422
prednisolone	100	5564 ± 3162*
-	100	4085 ± 1700
budesonide	100	2673 ± 1491 [#]

^a 1.25×10^6 monocytes in 1 ml culture medium were preincubated for 4 hr with either prednisolone (2×10^{-4} M) or budesonide (10^{-6} M), after which corticosteroids were washed away by three washes with culture medium. Four hr later LPS was added to the monocytes preincubated with corticosteroids or their respective controls (see Materials and Methods). After 13 hr supernatant was harvested for measurement of TNF concentration. Data represent the mean ± s.e.m. of 9 different individuals, each determined in triplicate.

* NS vs. control.

[#] $p < .05$ vs. control.

In conclusion, this study shows that the corticosteroids prednisolone and budesonide variably inhibit TNF secretion by monocytes, depending on the kind of stimulus used to induce TNF secretion. Strong inhibition was found when TNF secretion was induced by LPS or through FcγR cross-linking, weak inhibition was found with PMA-induced TNF secretion, and inhibition was intermediate with silica-induced TNF secretion. These differences may be of importance in explaining the variable effects of corticosteroids in several human disease states.

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EVIDENCE OF INVOLVEMENT OF TUMOR NECROSIS FACTOR IN ADVERSE REACTIONS DURING TREATMENT OF KIDNEY ALLOGRAFT REJECTION WITH ANTITHYMOCYTE GLOBULIN

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SUMMARY

Serial plasma concentrations of the pyrogenic cytokines tumor necrosis factor (TNF) and interleukin- 1β (IL- 1β) were measured during treatment of acute renal allograft rejection with antithymocyte globulin (ATG) in 7 consecutive kidney transplant recipients. TNF and IL- 1β were measured with specific enzyme-linked immunosorbent assays. In 6 of 7 patients TNF could not be detected in the plasma before the start of the ATG infusion. During the first ATG infusion, which was accompanied by fever and other side effects in all patients, plasma TNF levels were shown to be elevated, ranging between 100 and 700 pg/ml. During the second ATG infusion, when side effects were minimal or absent, plasma TNF levels were only slightly raised. Circulating IL- 1β could not be detected in any of the patients before or during ATG infusion. Additional experiments showed that TNF is rapidly secreted in cultures of peripheral blood mononuclear cells incubated with both ATG and the monoclonal antibody OKT3. These findings suggest that side effects, including fever and chills, during antilymphocyte antibody infusion are related to increased plasma levels of the pyrogenic cytokine TNF.

INTRODUCTION

A very effective treatment of acute kidney allograft rejection consists of intravenous administration of polyclonal rabbit or horse antilymphocyte antibodies (ALG) (1,2). More recently monoclonal anti-T cell antibodies like OKT3 are also used for this purpose (3,4). The application of both polyclonal and monoclonal antilymphocyte antibody therapy is in many cases severely hindered by the occurrence of serious side effects rendering it necessary to temporarily discontinue the antibody infusion. Side

effects include fever and chills, arthralgias, lumbar pain, nausea and vomiting, hypertension, tachycardia, angina pectoris, dyspnea caused by bronchospasm, and pulmonary edema (4-8). The application of ALG therapy can therefore be problematic in patients with concomitant pulmonary or cardiac disease.

The release of cytokines originating from lymphocytes lysed as a result of antibody infusion has been suggested to cause these side effects (3). Since fever and chills are such prominent symptoms seen in patients treated with antilymphocyte antibodies, we investigated whether plasma concentrations of the cytokines tumor necrosis factor/cachectin and interleukin- 1β (IL- 1β), which are known from animal experiments to possess strong pyrogenic activity (9), are elevated during treatment of kidney allograft rejection with antithymocyte globulin (ATG). Also, results of in vitro experiments elucidating the mechanism and cell source of ATG-induced TNF release are shown.

MATERIALS AND METHODS

Informed consent was obtained from seven consecutive kidney transplant recipients, treated for acute allograft rejection with antithymocyte globulin (ATG, Rijksinstituut voor de Volksgezondheid, Bilthoven, The Netherlands) to take serial blood samples for measurement of plasma cytokine concentration during ATG treatment.

ATG treatment consisted of intravenous administration of 100-300 mg ATG together with 50 mg prednisolone in 500 ml saline, infused over a 6-hr period. All patients received 4 mg of the antihistaminic chlorpheniraminemaleate orally 1/2 hr before start of the infusion. EDTA-anticoagulated 5-ml blood samples were drawn from an intravenous line inserted in the arm opposite the one in which ATG was infused. Samples were obtained at hourly intervals during the period of ATG infusion and once at a point after the end of ATG infusion when side effects, including fever, had completely disappeared. Blood samples were immediately centrifuged and aliquots of plasma were stored at -70°C for measurement of cytokine concentration. During treatment the patient's temperature and blood pressure were monitored and the occurrence of side effects was registered. Basic immunosuppression, which consisted of cyclosporine and low-dose prednisolone, was maintained during the study.

Enzyme-linked immunosorbent assay for TNF. Plasma TNF concentrations were determined with a TNF-specific ELISA, as described elsewhere (10). Flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with the monoclonal anti-TNF antibody 61E71 at a concentration of $5\text{ }\mu\text{g Ig/ml}$. Plates were blocked with 1% BSA in PBS for 1 hr at room temperature. Plasma samples were added to the plates in duplicate and incubated for 1 hr at room temperature. A standard titration curve was obtained by making serial dilutions of a known sample of recombinant TNF (rTNF; Celltech, Slough, UK) in EDTA-anticoagulated normal human plasma. After washing the plates four times, the amount of TNF bound to the wells was quantitated by sequential incubation with rabbit anti-human TNF immune serum and a peroxidase-conjugated goat anti-rabbit antibody (Jackson, West Grove, PA, USA; antibody not crossreactive with human serum proteins), followed by adding substrate to the wells and measurement of extinction at 495 nm with a microELISA autoreader. TNF-specific values were obtained by subtracting background extinctions due to non-TNF plasma proteins crossreacting in the ELISA, measured in the absence of either the monoclonal anti-TNF antibody coated on the bottom of the microtiter plate or the second, rabbit anti-TNF antibody. The lower detection limit of the ELISA was 5-10 pg/ml in plasma. Plasma samples of a large group ($n = 60$) of healthy volunteers were shown to be negative for TNF (i.e. less than 5 pg/ml) as determined with our assay. Plasma samples of patients were measured in duplicate and duplicate values always differed less than 10%.

Interleukin- 1β ELISA. Plasma levels of the endogenous pyrogen interleukin- 1β (IL- 1β) were measured in duplicate with a commercially available IL- 1β ELISA (kindly provided by Cistron, Pine Brook, NJ, USA). The ELISA proved to have a lower detection limit of 200 pg/ml IL- 1β in plasma.

In vitro experiments. Peripheral blood mononuclear cells (PBMC) were prepared by buoyant density centrifugation on Lymphoprep (Nycomed, Oslo, Norway) of buffy coats of donor blood kindly provided by the local Blood Bank. PBMC were cultured in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% heat-inactivated (56°C, 20 min) fetal calf serum (Boehringer, Mannheim, FRG) and 100 IU/ml penicillin and 100 µg/ml streptomycin (Flow, Irvine, UK). In each well of a 24-well plate (Greiner, Nürtingen, FRG) 2.5×10^6 PBMC were cultured in 600 µl culture medium. To these cultures various concentrations of ATG (for this study kindly provided by Dr. H. Kreeftenberg, Rijksinstituut voor de Volksgezondheid, Bilthoven, The Netherlands) and of the anti-CD3 monoclonal antibody OKT3 (ascites derived from an OKT3-secreting hybridoma, obtained from the ATCC, Rockville, MD, USA) were added and at different times samples of cell-free supernatant were harvested for measurement of TNF concentration. Lymphocyte proliferation was determined by measuring the incorporation of [3 H]-thymidine. For this purpose 0.5 µCi [3 H]-thymidine (Amersham, UK; specific activity 5.0 Ci/mmol) was added to a sample of 100 µl of the cell cultures transferred into round-bottomed microtiter plates (Greiner) and after a 4-hr culture period the radioactivity incorporated in the cells was measured with a liquid scintillation counter.

RESULTS

Case reports

Case 1. A 43-year-old man had had insulin-dependent diabetes mellitus for 27 years, which had led to renal insufficiency. Other long-term effects of diabetes in this patient were blindness due to diabetic retinopathy, hypertension, and intermittent claudication. On June 23, 1987, he received a cadaver donor kidney graft. After transplantation diuresis gradually increased and renal function improved. On the 18th postoperative day acute graft rejection was diagnosed on the basis of decreased urinary output, creatinine clearance, and graft perfusion. ATG treatment was started with the intravenous administration of 200 mg ATG and 50 mg prednisolone. Figure 1 shows the time curve of plasma TNF concentrations and body temperature during the first ATG infusion. Plasma TNF was detectable 1 hr after the start of the infusion. Plasma TNF rose to a peak level of 593 pg/ml at 3 hr and returned to a nondetectable level at 6 hr after start of the infusion. Body temperature rose to 39.4 °C 3 hr after TNF was detectable in the plasma. There was no significant change in systolic blood pressure, while diastolic blood pressure fell slightly during the period of peak TNF levels. ATG infusion was accompanied by chills, headache, diarrhea, and general malaise, which made it necessary to interrupt the infusion once. The patient received daily infusions of ATG for 16 days. Urinary output gradually increased, accompanied by a decline of serum creatinine from 717 to 323 µmol/L. After the ATG treatment immunosuppression was maintained with cyclosporine and low-dose prednisolone, and the patient was discharged from the hospital with a satisfactorily functioning renal allograft.

Case 2. This patient, a 54-year-old woman, had terminal renal insufficiency due to chronic pyelonephritis, that had required hemodialysis since March 1986. She also had hypertension, chronic bronchitis, polyneuropathy, and renal osteodystrophy. She received a cadaver donor kidney graft on June 13, 1987. Initially diuresis slowly increased, accompanied by a decrease in plasma creatinine from 1191 to 388 µmol/L, but on July 11 creatinine clearance diminished and histological examination of a renal biopsy showed interstitial rejection. The patient was treated with daily infusions of

Figure 1. Plasma TNF concentrations, body temperature, and blood pressure in case 1 during the first ATG infusion. Time scale marks hours after start of the ATG infusion. Plasma TNF concentrations are indicated by open circles, body temperature is indicated by closed circles. Systolic and diastolic blood pressures are indicated by downward pointing and upward pointing triangles, respectively.

ATG for 10 days. Figure 2 shows the time curve of plasma TNF levels and body temperature of this patient during the first ATG infusion. In this patient TNF rose to a maximal level of 731 pg/ml 3 hr after the start of the infusion, accompanied by a rise in body temperature to 40.4 °C 1 hr later. There was a rise in both systolic and diastolic blood pressure during the first few hours of ATG infusion. Side effects observed in this patient were severe dyspnea, which made it necessary to discontinue the ATG infusion twice, chills, headache, and general malaise. The ATG therapy was effective in reversing acute graft rejection. Urinary output increased and serum creatinine fell from 501 to 274 μ mol/L in the course of 4 weeks, after which the patient was discharged from the hospital. Immunosuppression was maintained with cyclosporine and low-dose prednisolone.

Plasma levels of TNF and IL-1 β in patients treated with ATG.

Using an assay with a lower detection limit of 200 pg/ml we could not detect circulating IL-1 β in any of the patients before or during ATG treatment. It remains, however, possible that IL-1 β at levels lower than 200 pg/ml was present in these

Figure 2. Plasma TNF concentrations, body temperature, and blood pressure in case 2 during the first ATG infusion. Symbols as in Figure 1.

patients, contributing to the fever and other side effects observed. In 6 of 7 patients pretreatment plasma TNF concentrations were below the detection limit of 5-10 pg/ml. In these patients TNF could be detected in the plasma approximately 1 hr after the start of the ATG infusion. Peak levels of TNF, ranging between 111 and 731 pg/ml, were reached after a 2-3 hr infusion period. Plasma TNF returned to an undetectable level at about 6 hr after the beginning of therapy. In only one patient (patient 2) was the plasma TNF concentration elevated before ATG was given (i.e. 132 pg/ml). In this patient plasma TNF rose to 179 pg/ml during ATG infusion and returned to a value of 112 pg/ml at the end of the infusion. All patients developed a rise in body temperature shortly after plasma TNF had reached peak values. Body temperature normalized on the average 4 hr after TNF disappeared from the circulation. In most of the patients a slight rise in blood pressure accompanied by an increased heart rate was observed during the ATG infusion. All patients experienced mild-to-severe side effects, which coincided in time with the period of elevated plasma TNF levels, and which made it necessary to temporarily discontinue the ATG infusion in several patients. In all patients symptoms waned shortly after plasma TNF had returned to normal. All

TABLE I. Plasma TNF concentrations and clinical data of ATG-treated patients

Patient (sex, age [years])	Treatment day	ATG (mg)	Highest TNFconc. (pg/ml)	Highest temp(⁰ C)	RR* (mm Hg)	Side effects
1. (M, 33)	1	300	560	39.4	+ 50/ + 5	Chills, vomiting
	2	200	55	37.9	0/0	None
2. (F, 54)	1	100	179	39.5	+ 10/0	Chills, dyspnea
3. (F, 35)	1	200	111	39.0	+ 5/ + 5	Chills
	2	200	24	38.0	0/0	None
4. (M, 49)	1	200	125	38.4	-30/-20	Chills, dyspnea, vomiting
	2	200	11	37.2	0/0	None
5. (F, 61)	1	200	138	38.2	+ 15/0	None
6. (F, 54)	1	200	731	40.4	+ 10/-10	Chills, dyspnea, headache
	2	100	0	37.4	0/0	None
7. (M, 43)	1	200	593	39.5	+ 15/-10	Chills, diarrhea, headache

*Expressed as difference between systolic and diastolic blood pressure measured before start of the ATG infusion and at the time of the highest plasma TNF concentration.

patients experienced a feeling of great fatigue at the end of the therapy. Table I gives the clinical data of all 7 patients. Only the first ATG infusion was accompanied by a sharp rise in plasma TNF level as well as by significant side effects. During the second ATG infusion, when side effects were considerably less, maximum TNF levels were much lower, i.e. between 0 and 55 pg/ml. Second-day plasma samples were not available from three patients.

Anti-T cell antibodies induce TNF production by peripheral blood mononuclear cells in vitro.

Both OKT3 and ATG, when added for 6 hr to cultures of PBMC, induced the secretion of TNF in a dose-related manner (Fig. 3). OKT3, which is a T cell mitogen, also induced T lymphocyte proliferation (Fig. 3A). ATG however did not induce T cell proliferation, indicating that proliferation of T lymphocytes is not a necessary prerequisite for TNF production in these cultures (Fig. 3B).

The kinetics of the ATG- and OKT3-induced release of TNF by PBMC was compatible with the rapid release of TNF in the circulation of the ATG-treated patients. Figure 4 shows that TNF concentration in the cell-free supernatant of these cultures rose rapidly after addition of ATG and OKT3 to the cultures, starting after about 2 hr. TNF concentration reached a plateau after a 5-6-hr culture period.

Figure 3. OKT3-induced (A) and ATG-induced (B) TNF secretion and cell proliferation in cultures of PBMC. TNF secretion and lymphocyte proliferation by PBMC after incubation with different concentrations of OKT3 or ATG were determined as described in the Methods section. Open circles denote TNF concentration in the supernatant after a 6-hr incubation period. Closed circles denote lymphocyte proliferation measured after a 3-day culture period. Data points represent means of triplicate determinations.

DISCUSSION

The treatment of acute rejection episodes, which occur in 40-60% of kidney allograft recipients despite immunosuppression with cyclosporine (11), has improved

Figure 4. Kinetics of OKT3- and ATG-induced TNF secretion by PBMC. PBMC were incubated with 10 ng/ml OKT3 (circles), 1 μ g/ml ATG (squares) or culture medium alone (triangles), and after various incubation periods TNF concentration in the supernatant was determined. Data points represent means of triplicate determinations.

significantly since the advent of antilymphocyte antibody therapy. A one-year graft survival rate of 78% has been reported for patients treated for acute graft rejection with rabbit antithymocyte globulin (5).

This report shows that treatment of acute graft rejection in kidney transplant recipients with intravenous administration of antilymphocyte antibodies leads to high plasma concentrations of the cytokine TNF, ranging between 100 and 700 pg/ml, during antibody infusion. TNF plasma concentration starts to rise between 30 min and 1 hr after the start of the ATG infusion, reaches peak levels after 3 hr, and declines to nondetectable levels in the course of the following hours, while ATG is still being infused. This indicates that TNF is released as a burst, triggered by the infused ATG. Preliminary data show that infusion of the monoclonal anti-T cell antibody OKT3 into humans also leads to raised plasma TNF levels (data not shown).

Since TNF can induce the production of another group of important endogenous pyrogens, IL-1 (9), we also measured IL-1 levels. On the basis of different isoelectric focusing patterns two forms of IL-1 are distinguished, IL-1 α and IL-1 β , which have very similar biological effects. We measured IL-1 β levels in our patients because of the availability of an IL-1 β immunoassay. We were not able to detect IL-1 β in the plasma of any of the patients before or during ATG treatment. It remains however possible, considering the detection limit of the IL-1 β immunoassay, that levels of IL-1 β lower than 200 pg/ml were present in the patients, contributing to the fever and other systemic reactions observed in these patients.

The majority of patients, 6 of 7, had no detectable TNF plasma levels before start of the ATG treatment. In only one patient (patient 2), who at that time had no fever, was

the pretreatment plasma TNF level elevated (132 pg/ml). In this patient there was only a slight rise in plasma TNF level to 179 pg/ml during the ATG infusion; nevertheless it was accompanied by fever, chills, and dyspnea. Others, however, detected elevated serum TNF levels in the majority of kidney transplant recipients during acute rejection episodes (12). These different findings might be due to differences in the severity of kidney allograft rejection or to differences in the time during allograft rejection when plasma or serum samples for measurement of TNF levels were obtained.

The occurrence of systemic reactions to the ATG infusion correlates with the period of elevated plasma TNF levels, which suggests that side effects are related to or even caused by raised plasma TNF levels. This is further substantiated by data from phase I studies of human recombinant TNF in the treatment of cancer, which show that side effects observed in patients treated with recombinant TNF are remarkably similar to those seen in patients receiving ALG for treatment of graft rejection. Side effects observed in both patient groups include fever, chills, nausea, vomiting, diarrhea, arthralgias, headaches, and fatigue (13,14).

TNF is a cytokine with cytotoxic and immunoregulatory properties (15), that is primarily produced by activated monocytes and macrophages (16,17), but also, as has recently been shown, by activated T lymphocytes (18). The appearance of TNF in the circulation of patients treated with ATG is possibly due to partly complement-mediated intravascular lysis of activated T lymphocytes. Monocytes and macrophages are known to secrete inflammatory mediators after opsonization of antibody-coated cells (19). We have recently demonstrated that TNF is also secreted by human monocytes after Fc-receptor cross-linking occurring during opsonization and phagocytosis of antibody-coated sheep erythrocytes (20). It is therefore conceivable that in the process of opsonization of ATG-coated lymphocytes TNF is released by circulating monocytes. Our *in vitro* experiments support the latter hypothesis. In these experiments TNF is rapidly secreted by peripheral blood mononuclear cells after incubation with ATG and OKT3 without complement-mediated lysis of T lymphocytes, since the experiments were performed in the presence of decapitated serum. Since activated T lymphocytes have been shown to produce TNF (18), we cannot rule out that in the case of OKT3 TNF is, at least in part, produced by activated T lymphocytes.

TNF has recently been implicated as a host mediator responsible for shock and tissue damage induced by endotoxin in the course of septic shock. Evidence supporting this hypothesis has primarily come from animal experiments (21). Mice passively immunized against murine TNF were shown to be largely resistant to a lethal dose of endotoxin (22), whereas baboons could be protected from the lethal effects of intravenous injection of live bacteria by prior administration of anti-human TNF antibodies (23). Recently also clinical data have been published that assign an important role to TNF in the pathogenesis of septic shock in man (24). Circulating TNF has been demonstrated in healthy volunteers after injection of endotoxin (25). Our data show that patients can tolerate high levels of endogenously produced TNF without the occurrence of hypotension or shock, indicating that in sepsis other mediators besides TNF probably are involved in the pathogenesis of shock.

Besides its role in sepsis TNF possibly contributes to the host defence mechanisms against cancer. Its value as an anti-cancer drug is currently being investigated (13,14). Interestingly, TNF-like factors have been discovered in the serum of cancer patients (24).

In conclusion, the data from this study indicate that systemic reactions accompanying treatment of kidney allograft rejection with antilymphocyte antibodies are related to high circulating levels of TNF, although a contribution by other (pyrogenic) endogenous mediators like IL-1, IL-6, lymphotoxin, and IFN- α cannot be excluded.

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PLASMA TUMOR NECROSIS FACTOR AND MORTALITY IN CRITICALLY ILL SEPTIC PATIENTS

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SUMMARY

Tumor necrosis factor/cachectin has been implicated as an important host mediator responsible for shock and multiple organ failure observed during sepsis. Using a sensitive enzyme-linked immunosorbent assay, we measured plasma TNF levels in 43 septic patients suffering from a broad range of diseases. Measurements were taken on the day that sepsis was diagnosed. Eleven patients had detectable TNF plasma levels ranging from 10 to 100 pg/ml (TNF-positive group); in 32 patients circulating TNF could not be detected (TNF-negative group). The groups did not differ significantly as to age, underlying disease, percentage positive bacteremia and bacteriologic profile, sepsis score, and extent of MOF. Eight (73%) of 11 TNF-positive patients died from sepsis during ICU stay, vs. 11 (34%) of 32 TNF-negative patients ($p < .05$). This study demonstrates that sepsis is accompanied by detectable circulating TNF in 25% of the cases, and for these patients mortality is twice that for comparable TNF-negative patients.

INTRODUCTION

In recent years, evidence has accumulated that endogenous mediators play an essential role in the pathogenesis of shock and multiple organ failure (MOF) during sepsis. Circulatory instability during gram-negative bacteremia, originally attributed to circulating endotoxins, is now believed to be caused by cytokines produced by cells of the immune system in response to generalized infection. The cytotoxic and immunoregulatory cytokine tumor necrosis factor (TNF)/cachectin is considered to be one of the prime mediators of the host response to endotoxin involved in the pathophysiology of sepsis (1). Although knowledge on TNF as an inflammatory and immunoregulatory mediator has been gained from experimental studies (2), clinical data on TNF are still scarce.

We investigated the role of TNF in human sepsis by measuring plasma TNF levels in a series of septic ICU patients using a sensitive enzyme-linked immunosorbent assay (ELISA) developed in our laboratory. Plasma samples were drawn whenever a severe septic episode occurred. Patients were allocated to TNF-positive or TNF-negative groups depending on whether or not circulating TNF was detectable at the time of sepsis. Both groups were compared as to the underlying disease, presence of bacteremia, severity of sepsis, and severity of MOF. We report a positive correlation between circulating TNF and mortality in septic patients suffering from a diverse range of diseases.

PATIENTS AND METHODS

Patients. Forty-three patients (32 men and 11 women, age 19 to 94 years) admitted to the ICU of Maastricht University Hospital during a 10-month period were studied. All patients, suffering from a wide range of diseases, underwent a septic episode during their ICU stay. Sepsis was diagnosed when one or more of the following criteria were met: fever $>38^{\circ}\text{C}$ with 1) a drop in systolic BP >30 mm Hg, 2) urine output <20 ml/hr during more than 2 hr, 3) platelet count $<100 \times 10^6/\text{ml}$, 4) plasma bicarbonate <15 mmol/l. On the day that sepsis was diagnosed, one blood sample was collected from each patient for measurement of plasma TNF level. These blood samples were always collected together with the first daily round of routine blood sampling at approximately 8 AM. Five-milliliter EDTA-anticoagulated blood samples were obtained and centrifuged for 5 min at 3000 rpm. Plasma was stored in divided aliquots at -70°C for measurement of TNF concentration at a later date.

Patients were divided into two groups: patients in whom TNF could be detected in the plasma at the time of sepsis (TNF-positive group) and patients in whom circulating TNF could not be detected (TNF-negative group). Both groups were evaluated for age, sex, underlying disease, severity of sepsis, severity of MOF, and presence of bacteremia. All these parameters refer to the patient's condition during the first 24 hr after onset of sepsis.

Severity of sepsis was estimated by the sepsis score developed by Elebute and Stoner (3). This score comprises a simple system for grading the severity of sepsis by scoring the attributes of sepsis under four headings: local effects of infection, which is the major determinant of the score, pyrexia, secondary effects of sepsis, and laboratory data. Each attribute is scored with 0, 1, 2, or 3 points (except for the local effects of infection, which are scored double) and the sum of all the scores gives an aggregate criterion representing the total effect of the septic state on the patient.

Extent of MOF was determined by measuring mean blood pressure (BP) and a series of laboratory parameters estimating basic organ function. Pulmonary function of patients receiving ventilatory support was evaluated by measuring arterial oxygen tension divided by fractional O_2 content of the inspired air ($\text{PaO}_2/\text{FIO}_2$); liver function was estimated by SGPT, SGOT and LDH; renal function was estimated by serum creatinine, and extent of disseminated intravascular coagulation was evaluated by platelet count and presence of fibrinogen degradation products (FDP). All these parameters represent optimal values obtained during 24 hr of intensive treatment after the onset of sepsis, thereby indicating both severity of disease and the patient's response to therapy.

Enzyme-linked immunosorbent assay (ELISA) for TNF. Plasma TNF concentrations were determined with a modified TNF-specific ELISA, which is described elsewhere (4). In short, patient plasma samples were added onto 96-well immunoassay plates coated overnight with $0.5 \mu\text{g}/\text{well}$ of the monoclonal anti-TNF antibody 61E71. Before adding test samples, the plates were blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 hr at room temperature. A standard titration curve was obtained by making serial dilutions of a known sample of human recombinant TNF (kindly provided by Celltech, Slough, UK) in normal human plasma. The amount of TNF bound to the wells was quantified by sequential incubation with a 1:2000 dilution of rabbit anti-TNF immune serum and a 1:1000 dilution of peroxidase-conjugated goat anti-rabbit antibody (Jackson, West Grove, PA; antibody not

crossreactive with human serum proteins), followed by adding substrate (OPD; Sigma, St. Louis, MO) to the wells. The color reaction was stopped with 1.0 M H₂SO₄, after which extinction at 495 nm was measured with a microelisa autoreader (Flow, Irvine, UK). TNF-specific values were obtained by subtracting background absorptions due to non-TNF plasma proteins crossreacting in the ELISA, measured in the absence of either the monoclonal anti-TNF antibody coated on the bottom of the ELISA plate or the second, rabbit anti-TNF antibody. The lower detection limit of the ELISA ranged from 5-10 pg/ml. Plasma samples of a large group (n = 60) of healthy volunteers were shown to be negative for TNF as determined with our assay.

Statistical analysis. Student's t-test for independent groups and chi-square test without Yates' correction were used for statistical analysis of the data. Data were considered to be significantly different at the $p < .05$ level.

RESULTS

Forty-three plasma samples were obtained from the patients on the day sepsis was diagnosed. Eleven patients had detectable plasma TNF levels, ranging from 10 to 100 pg/ml (TNF-positive group). In 32 patients, no circulating TNF could be detected (TNF-negative group). Mean ages of TNF-positive and TNF-negative patients (60.7 yr and 61.7 yr respectively) did not differ significantly. Patient characteristics and plasma TNF levels are described for both TNF-positive (Table I) and TNF-negative patients (Table II).

TABLE I. Patient characteristics of TNF-positive patients

	Sex	Age	Major illness	Culture	Sepsis score	TNF (pg/ml)
1.	m	43	Acute laryngitis	bl: <i>H. influenzae</i>	10	15
2.	m	84	Pneumonia after aortic aneurysm operation	sp: <i>Acinetobacter calc.</i>	13	36
3.	m	67	Pneumonia and lung carcinoma	sp: <i>S. pneumoniae</i>	9	45
4.	m	79	Peritonitis after gastric ulcer perforation	sp: <i>C. albicans</i>	13	36
5.	f	97	Pyelonephritis, pneumonia	bl: <i>S. aureus</i>	19	122
6.	m	94	Pneumonia	bl: <i>Klebsiella oxytoca</i>	13	22
7.	f	19	Head injury, pneumonia	sp: <i>S. aureus</i>	10	27
8.	f	48	SLE, dyspnea, sepsis of unknown origin	bl: <i>S. aureus</i>	21	23
9.	m	68	Chronic bronchitis, pneumonia	sp: <i>Acinetobacter calc.</i>	10	27
10.	m	53	Wound infection and pneumonia after coronary artery surgery	bl: <i>C. albicans</i> , <i>S. aur.</i>	20	78
11.	m	56	Pelvic abscess after hemicolectomy and aortic aneurysm	not available	11	30

bl: blood; sp: sputum.

TABLE II. Patient characteristics of TNF-negative patients

Sex	Age	Major illness	Culture	Sepsis score
1. m	77	Uretercarcinoma, peritonitis after urinary bladder necrosis	bl:Klebsiella pneumoniae	15
2. f	71	Femoral fracture, pneumonia	bl:S.aureus	19
3. m	83	Peripheral vascular occlusion, pneumonia	sp:Acinetobacter,E. coli	13
4. m	22	Severe trauma, wound infection	pus:Acinetobacter,E. coli	16
5. f	66	Pelvic abscess, adnexitis, sigmoiditis	pus:Morganella morganii, S. faecalis	16
6. m	49	Peritonitis, hepatic abscess	bl:gram-negative rods	10
7. f	53	Chronic bronchitis, pneumonia	bl:S. pneumoniae	11
8. m	72	Cholangitis	bl:E. coli	16
9. f	68	Urinary tract infection	bl:E. coli	18
10. m	70	Pneumonia, urinary tract infection	sp:H. influenzae ur:E. coli	9
11. m	44	Empyema, pneumonia	sp:S. epidermidis	16
12. m	46	Pneumonia after severe trauma	bl:S. epidermidis,C. alb.	17
13. f	70	Diabetes mellitus, pneumonia	sp:S. aureus,H. influenzae	18
14. f	71	Aspiration pneumonia	sp:E. coli	13
15. m	73	Pneumonia	bl:Klebsiella pneum.	21
16. f	84	Pelvic abscess, coloncarcinoma	bl:S. epidermidis	18
17. m	67	Esophageal varices, aspiration pneumonia	sp:E. coli,Acinetobacter	17
18. m	67	Myocardial infarction, fever of unknown origin	sp:P. aeruginosa	12
19. m	66	Guillain-Barré, pneumonia	sp:H. influenzae	12
20. m	80	Cholecystectomy, pneumonia	bl:S. epidermidis	13
21. m	30	Subphrenic abscess after Mason procedure	pus:S. aureus	11
22. m	22	Autointoxication, pneumonia	sp:S. pneum.,H. influenz.	10
23. m	72	Pneumonia	bl:Acinetobacter,S. epiderm.	13
24. m	45	Traumatic splenic rupture	bl:S. epidermidis	23
25. m	78	Urinary tract infection	bl:Klebsiella pneumoniae	22
26. m	58	Aspiration pneumonia	sp:S. hemolyt.,H. influenzae	8
27. m	71	Fever after aortic aneurysm	not available	12
28. m	77	Rectumcarcinoma, peritonitis after low anterior resection	bl:S. aureus	23
29. m	58	Aortic aneurysm, wound infection, pneumonia	sp:C. albicans;pus:E. coli	16
30. m	92	Femoral hernia, pneumonia	sp:E. coli	12
31. f	72	Peritonitis after laparotomy	bl:S. aureus	16
32. m	52	Laryngeal carcinoma, wound infection	pus:Enterobacter,Bacteroides	11

bl: blood; sp: sputum; ur: urine.

Figure 1. Sepsis score of TNF-negative and TNF-positive patients. Open circles: survivors; closed circles: nonsurvivors.

A significant correlation existed between sepsis score and mortality for the TNF-negative group ($p < .05$), as well as for both groups combined ($p < .05$) (Fig. 1). The mean sepsis score was 13.3 for the TNF-positive group and 14.9 for the TNF-negative group ($p = .18$).

The severity of organ failure for TNF-positive and TNF-negative patients is outlined in Table III. The parameters given in Table III represent optimal values obtained during a 24-h period after the onset of sepsis, during which each patient was intensively treated. These data, which were always obtained on the same day plasma TNF levels were determined, thereby reflect both severity of disease and the patients' response to therapy. No significant difference was found between both groups for mean BP, pulmonary shunting, liver and kidney function tests, and platelet count. Circulating FDP were measured in seven TNF-positive and in 21 TNF-negative patients. FDP were present in six of seven TNF-positive and in 19 of 21 TNF-negative patients.

According to the clinical findings on the day plasma samples were taken, the patients were classified into five disease categories based on the most likely focus of infection causing the sepsis. Twenty-one patients had respiratory tract infection, ten patients peritonitis or pelvic abscess, four urinary tract infection, four wound infection, and of four patients the focus of infection remained unknown. TNF-positive and TNF-negative patients were equally distributed among these categories (Table IV).

Blood cultures obtained on the first day of sepsis from 20 patients were found to be positive: five in the TNF-positive group (45% of TNF-positive patients) and 15 in the TNF-negative group (47% of TNF-negative patients; $p = .79$). The cause of bacteriologically proven sepsis was a Gram-negative microorganism in 44% of TNF-positive

TABLE III. Severity of multiple organ failure in TNF-positive and TNF-negative septic patients

	TNF-positive patients	TNF-negative patients	p
MBP (mmHg)	98.3	95.9	0.31
PaO ₂ /FIO ₂ * (kPa)	34.9	27.0	0.07
SGPT (U/l)	60.6	63.7	0.46
SGOT (U/l)	85.6	96.1	0.21
LDH (U/l)	768.5	896.8	0.32
Creatinine (μmol/l)	134.5	177.9	0.23
Platelets (10 ⁶ /ml)	158	239	0.07

MBP = mean blood pressure.

*Seven of 11 TNF-positive and 22 of 32 TNF-negative patients received ventilatory support.

TABLE IV. Relation between site of infection, positive blood cultures, and TNF-positive plasma samples in septic patients

Disease category	Total	Positive blood culture	Survived	Died	TNF positive
Respiratory tract infection	21	8	11	10	6
Peritonitis/pelvic abscess	10	6	6	4	2
Urinary tract infection	4	3	3	1	1
Wound infection	4	1	2	2	1
Unknown focus	4	2	2	2	1
Total	43	20	24	19	11

TABLE V. Relation between plasma TNF and mortality in septic patients

	TNF positive	TNF negative	Total
Patients died	8	11	19
Patients survived	3	21	24
Total	11	32	43

$p < .05$.

patients and 68% of TNF-negative patients ($p = .22$). Patients received antibiotics at the time plasma samples were taken in 70% of the cases (64% in the TNF-positive group and 72% in the negative group; $p = .61$).

Nineteen patients eventually died from sepsis during ICU stay. Of these, eight patients were in the TNF-positive group and 11 in the TNF-negative group. The mortality rate during the ICU stay was 73% for the TNF-positive group and 34% for the TNF-negative group (Table V). This difference was statistically significant ($p < .05$).

DISCUSSION

Sepsis is observed frequently in patients admitted to ICU departments, leading to morbidity and mortality. Sepsis can present itself in many different ways, affecting virtually any organ or organ system. Fever, hypotension, cardiorespiratory insufficiency, the adult respiratory distress syndrome, and renal insufficiency are common manifestations of sepsis in man (5).

Sepsis is believed to be caused by several bacterial products, most notably lipopolysaccharides (LPS) derived from the cell wall of Gram-negative microorganisms infecting the host. In recent years, data have accumulated indicating that host derangements observed during sepsis are caused by endogenous mediators produced by cells of the immune system in response to overwhelming infection.

TNF/cachectin is believed to be one of the primary mediators involved in the pathophysiology of sepsis (1). It is produced by monocytes/macrophages in response to LPS, but Gram-positive bacterial products also have been shown to induce TNF production in vitro (6). TNF is an endogenous pyrogen, causing fever in experimental animals (7) and in man (8). TNF, when administered at high doses to experimental animals, causes abnormalities indistinguishable from those observed during sepsis (9-11). Moreover, it has been shown that mortality due to experimental sepsis in mice (12) and baboons (13) can be prevented by prior administration of neutralizing anti-TNF antibodies, indicating that in these experimental models TNF is a crucial intermediate in the downhill course of sepsis, culminating in shock and death.

In man, raised plasma TNF levels have been found after administration of endotoxin to normal volunteers (14). However, only one study has until now shown that

raised serum TNF levels negatively affect outcome in septic patients (15). The patients in this study were suffering from meningococcal meningitis complicated with sepsis, a disease entity which is not frequently encountered in general ICU populations.

In the present study, we examined an unselected group of septic patients suffering from a diverse range of diseases underlying the septic state. On the day sepsis was clinically diagnosed, the presence of circulating TNF was determined. On this basis, patients were allocated to TNF-positive or TNF-negative groups. Both groups were comparable with regard to underlying disease, percentage of established bacteremia, bacteriological profile, sepsis score, and extent of MOF. However, the mortality in the TNF-positive group was twice the mortality in the TNF-negative group. Apparently, the presence of TNF at the time of sepsis indicates a decreased chance of survival.

Circulating TNF could be detected in one of four septic patients. This might be due to differences in levels of bacteremia/endotoxemia or, otherwise, to interindividual variation in the biological response to sepsis. Preliminary data from our laboratory indicate that there exists, as previously shown for the cytokine interleukin-1 (16), a great variability in the endotoxin-induced production and secretion of TNF amongst different individuals. Whether this means that TNF-positive patients in our study consist of 'high responders', who therefore are more at risk of developing shock and MOF during sepsis, remains to be clarified. It might also be possible, since a burst-like release of TNF, circulating with a short half-life, has been shown in man after endotoxin administration (14), that the time point at which we obtained blood samples was not always at the peak of TNF release in our patients.

Waage *et al.* (15), using a TNF bioassay, found in patients suffering from meningococcal meningitis and sepsis TNF plasma values ranging from 3 pg/ml to 36 ng/ml. TNF levels above 100 pg/ml were associated with 100% mortality. TNF levels in our TNF-positive patients were lower, ranging from 10 to 100 pg/ml. This might be due to differences in endotoxin levels, which are known to be very high in meningococcal sepsis. Due to the restricted number of TNF-positive samples, the correlation between plasma TNF concentration and mortality in our patients could not be evaluated.

Data from experimental studies indicate that TNF/cachectin is involved in the etiology of cachexia accompanying infectious and malignant diseases (17, 18). Our finding that one of four septic patients had detectable plasma TNF levels suggests that TNF/cachectin may also contribute to the cachexia which is frequently observed in septic ICU patients.

Our data substantiate a correlation between circulating TNF and mortality in septic patients suffering from a broad range of underlying diseases. Further study must establish whether the presence of circulating TNF during sepsis is merely a prognostic sign indicating poor patient survival, or whether TNF plays a pivotal role in the initiation of shock and MOF. In the latter instance, intervention with anti-TNF antibody therapy would offer potential therapeutic opportunities in these patients.

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SUMMARY

Tumor necrosis factor (TNF)/cachectin is a cytokine released by immunocompetent cells involved in inflammatory processes. Numerous studies indicate that it is a major acute phase reactant involved in the host response to infection and trauma. TNF exerts widespread effects on many different cell types and tissues, influencing inflammatory, hemostatic, hormonal and metabolic processes. TNF also possesses strong cytotoxic and antiviral properties, thus probably constituting an important effector cytokine in the host response against viral and malignant disease. Besides its physiological role TNF is considered to be involved in the pathogenesis of several disease states like sepsis, and cachexia accompanying infectious and malignant diseases.

In this thesis both *in vitro* and *in vivo* studies on the cytokine tumor necrosis factor are presented. The results from *in vitro* experiments, studying various aspects of the regulation of production and secretion of TNF by human monocytes, are presented in chapters 2 to 5. Chapters 6 and 7 contain results from *in vivo* studies on the role of circulating TNF in two different patient populations.

In chapter 2 experiments are described which demonstrate that T cell mitogens induce TNF production by peripheral blood mononuclear cells in a T cell-dependent manner. Although it has been demonstrated by other investigators that activated T lymphocytes are capable of producing TNF, TNF is shown to be produced predominantly by monocytes under the experimental conditions of our study. In this study it is also demonstrated that the T cell lymphokine interferon- γ (IFN- γ) is an important humoral mediator of this T cell-dependent pathway of TNF production by monocytes, being capable of inducing TNF secretion by monocytes in endotoxin-free circumstances. This finding confirms data from murine experiments, in which it was demonstrated that IFN- γ enhances macrophage TNF synthesis at a transcriptional level. However, in contrast with our findings with human monocytes, secretion of TNF protein by murine macrophages is dependent on the presence of endotoxin. The findings of this study implicate that, in man, TNF production will not only occur during immune responses against infectious diseases, but also during aseptic immune and inflammatory processes accompanied by activation of the T lymphocyte system.

In chapter 3 it is demonstrated that cross-linking of the receptors for the Fc domain of IgG (Fc γ R) strongly stimulates TNF secretion by human monocytes. The Fc γ R plays an important role in the interaction between the humoral and cellular immune systems, being involved in the mediation of phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), induction of secretion of inflammatory mediators by immune cells, and clearance of potentially harmful immune complexes. Our finding that TNF

secretion by monocytes is strongly stimulated by Fc γ R cross-linking has several implications as to the role of TNF in these immune processes. First, TNF is considered to be an important mediator of monocyte/macrophage cytotoxicity. Our finding that monocyte TNF secretion is induced by Fc γ R-ligand interactions makes it conceivable that TNF is an important effector molecule of monocyte/macrophage ADCC. Since TNF has been shown to possess antiviral and antiparasitic properties, these findings also suggest its involvement in phagocytic processes during viral and parasitic infections. Finally, TNF has been shown to exert detrimental effects on various normal cells and tissues. It might thus be involved in the pathogenesis of tissue destruction occurring in the course of immune complex diseases like rheumatoid arthritis and (antibody-mediated) autoimmune diseases like type I diabetes mellitus.

The role of the two constitutively expressed monocyte Fc γ R in the regulation of TNF secretion has been studied more elaborately in chapter 4. It is demonstrated that on freshly isolated, untreated monocytes, only cross-linking of the 72 kDa, high-affinity Fc γ R, Fc γ RI, triggers TNF secretion, whereas cross-linking of a second class of Fc γ R, the 40 kDa Fc γ RII, does not lead to TNF secretion. However, after treatment of monocytes with proteolytic enzymes or with the desialylating enzyme neuraminidase, Fc γ RII gains capacity to induce TNF secretion. It is demonstrated that the capacity of Fc γ RII to induce TNF secretion after enzyme treatment of monocytes is related to an increase in affinity of this receptor for Fc ligand. Moreover, Fc γ R-mediated TNF secretion is shown to be dependent on Fc-Fc γ R interactions, TNF secretion being absent when Fc γ R are cross-linked with anti-Fc γ R monoclonal antibodies, which interact with Fc γ R via their antigen-binding site. This study suggests a differential role of both Fc γ R in the regulation of TNF secretion by monocytes. Furthermore, the findings of this study make it conceivable that TNF secretion is limited to areas of active inflammation, thus protecting non-inflamed areas against its potential harmful effects.

In chapter 5 we have studied the effect of corticosteroids, which have strong immunosuppressive and antiinflammatory properties, on monocyte TNF secretion induced by four different stimulating agents. Corticosteroids inhibit, as has also been shown for various other inflammatory mediators, TNF secretion. The degree of inhibition is, however, dependent on the type of stimulus used to induce TNF secretion. TNF secretion induced by endotoxin and through Fc γ R cross-linking is shown to be strongly inhibited, whereas TNF secretion following protein kinase C activation is only poorly inhibited. Inhibition of silica-induced TNF secretion is intermediate in comparison with LPS/Fc γ R-induced and PMA-induced TNF secretion. Furthermore, sustained inhibition of TNF secretion requires the continual presence of corticosteroids, inhibition being reversible after removal of corticosteroids from the cultures. These findings explain yet another mechanism by which corticosteroids exert their immunosuppressive and antiinflammatory effects. Since TNF is considered to be a key mediator of endotoxic shock and sepsis, this finding offers a possible explanation for the protective effect of corticosteroids observed in experimental sepsis studies in animal models. This study also clarifies one of the mechanisms underlying the therapeutic efficacy of corticosteroids in immune complex diseases like rheumatoid arthritis, in which TNF is believed to be pathogenetically involved.

In chapter 6 it is demonstrated that treatment of kidney allograft rejection with antithymocyte globulin (ATG) is accompanied by a sudden, short-lived, rise in circulating TNF levels. Moreover, side effects observed during this treatment are shown to occur concurrently with elevated plasma TNF levels, which suggests that TNF is involved in the causation of these symptoms. This conclusion is substantiated by findings of phase I clinical trials with recombinant TNF as an anticancer drug. Side effects observed during administration of rTNF to humans are remarkably similar to those we observed in our patients treated with ATG. It seems clear from this study that under non-septic circumstances raised plasma TNF levels are by themselves not sufficient to cause lethal shock and widespread organ damage, as has been suggested by findings from animal experiments. In vitro studies indicate that TNF release induced by ATG is not due to lysis or activation of T lymphocytes. Possibly, TNF is secreted by monocytes/macrophages upon opsonization of ATG-coated T lymphocytes via a Fc receptor-mediated process, as has been described in chapter 3.

Finally, in chapter 7 it is demonstrated that in septic patients circulating TNF can be detected in one of four patients. It is shown that in septic patients with circulating TNF at the time of sepsis, mortality is twice the mortality of patients without circulating TNF. The difference in mortality between patients with or without circulating TNF seemed not to be caused by differences in degree of sepsis or extent of multiple organ failure, since parameters used to estimate the severity of sepsis and multiple organ failure did not differ significantly between both patient groups. Numerous experimental animal studies have implicated TNF as a crucial endogenous mediator involved in the pathogenesis of sepsis and septic shock. Two clinical studies have demonstrated a relation between the presence of circulating TNF and mortality during meningococcal sepsis. Our study corroborates these findings and extends them to patients suffering from a broad range of diseases underlying the septic state.

SAMENVATTING

Het immuunsysteem van de mens heeft als doel het menselijk organisme te beschermen tegen infecties met pathogene micro-organismen, zoals bacteriën, virussen, parasieten en schimmels. Daarnaast speelt het immuunsysteem een belangrijke rol bij het voorkomen van het ontstaan van kwaadaardige gezwellen. Het goed functioneren van het immuunsysteem vereist een complexe samenwerking tussen vele verschillende soorten cellen, zowel van hematopoietische als niet-hematopoietische oorsprong. Voor de onderlinge communicatie maken deze cellen gebruik van cytokines. Dit zijn moleculen, veelal proteïnen, die door immuuncompetente cellen gesecreteerd worden tijdens immuun- en ontstekingsprocessen. Deze cytokines kunnen een regulerend effect uitoefenen op dezelfde cellen waardoor ze geproduceerd zijn (autocriene beïnvloeding), op andere cellen in de onmiddellijke omgeving (paracriene beïnvloeding), of op cellen elders in het lichaam (endocriene beïnvloeding).

Tumor necrosis factor (TNF), ook wel cachectin genaamd, is een van deze cytokines. TNF is een 17 kDa eiwit, dat in de mens voornamelijk wordt geproduceerd door mononucleaire leukocyten, i.e. monocyten, macrofagen, en lymfocyten. TNF productie door monocyten en macrofagen wordt zeer krachtig gestimuleerd door het lipopolysaccharide (LPS) van gram-negatieve bacteriën (endotoxine). TNF heeft een groot aantal immunoregulatorische en inflammatoire eigenschappen en vormt een belangrijke endogene mediator betrokken bij de acute fase respons tijdens infecties en bij trauma. TNF bezit tevens cytotoxische en antivirale eigenschappen en vormt daardoor een belangrijk effector proteïne van de immuunrespons tegen maligniteiten en virale infecties. Naast een fysiologische rol zijn er aanwijzingen dat TNF ook betrokken is bij de pathogenese van verschillende ziekteprocessen, met name bij sepsis en septische shock, en bij cachexie optredend tijdens infecties of maligniteiten.

In dit proefschrift worden *in vitro* en *in vivo* studies van het cytokine TNF beschreven. Hoofdstuk 1 geeft een algemene inleiding en een overzicht van de literatuur met betrekking tot de fysiologische en pathofysiologische betekenis van TNF. Hoofdstuk 2 geeft een inleiding tot en bespreking van het experimentele werk beschreven in dit proefschrift. *In vitro* studies, die verschillende aspecten van de regulatie van TNF productie en secretie door humane monocyten als onderwerp hebben, worden beschreven in hoofdstuk 3 tot en met 6. Hoofdstuk 7 en 8 bevatten studies naar de *in vivo* rol van circulerend TNF bij twee verschillende patiëntenpopulaties.

In hoofdstuk 3 worden experimenten beschreven die aantonen dat T cel mitogenen op een T cel-afhankelijke wijze TNF secretie door mononucleaire cellen induceren. Hoewel andere onderzoekers hebben aangetoond dat geactiveerde T lymfocyten TNF

kunnen produceren, wordt TNF onder de experimentele condities van onze studie voornamelijk geproduceerd door monocysten. In deze studie wordt ook aangetoond dat het door T lymfocyten geproduceerde cytokine interferon- γ (IFN- γ) een belangrijke humorale mediator is van deze T cel-afhankelijke TNF productie door monocysten. IFN- γ induceert dosis-afhankelijk TNF secretie door monocysten. Deze bevinding bevestigt de resultaten van andere onderzoekers, die in muizestudies aantoonden dat IFN- γ TNF productie door macrofagen op transcriptieniveau verhoogt. In tegenstelling echter tot deze studies, waarin TNF secretie alleen maar plaatsvindt in de aanwezigheid van endotoxine, wordt in onze studie TNF secretie door monocysten ook geïnduceerd door IFN- γ in de afwezigheid van endotoxine. De bevindingen van onze studie impliceren dat in de mens TNF productie en secretie niet alleen tijdens infectieuze ziektes zal optreden, maar ook tijdens niet-infectieuze immuun- en ontstekingsprocessen waarbij het T cel systeem wordt geactiveerd (bv. transplantaatrejecties, autoimmuunziektes etc.).

In hoofdstuk 4 wordt aangetoond dat cross-linking van de receptor voor de Fc staart van IgG (Fc γ receptor) TNF secretie door monocysten induceert. Fc γ receptoren spelen een belangrijke rol bij de interactie tussen het humorale en het cellulaire immuunsysteem. Fc γ receptoren zijn van belang voor verschillende immuunprocessen, zoals fagocytose, antilichaam-afhankelijke cellulaire cytotoxiciteit (ADCC), de inductie van secretie van ontstekingsmediatoren door immuuncompetente cellen, en de klaring van potentieel schadelijke immuuncomplexen. Onze bevinding dat TNF secretie door monocysten in sterke mate gestimuleerd wordt door cross-linking van Fc γ receptoren, heeft verschillende implicaties met betrekking tot de rol die TNF in deze immuunprocessen speelt. TNF wordt beschouwd als een mediator van de cytotoxische activiteit van monocysten en macrofagen, bijvoorbeeld gericht tegen tumorcellen. TNF vormt waarschijnlijk een belangrijk effector molecuul in de afweer tegen maligne processen. Fc γ receptor-geïnduceerde TNF secretie vormt een mogelijke verklaring van het mechanisme van TNF productie tijdens ADCC gericht tegen maligne cellen. Daarnaast bezit TNF antivirale en antiparasitaire eigenschappen. Tijdens een humorale immuunrespons tegen viraal geïnfecteerde cellen of tegen parasieten zal TNF secretie worden geïnduceerd via cross-linking van Fc γ receptoren op monocysten/macrofagen betrokken bij de immuunrespons. TNF secretie door monocysten blijkt ook zeer krachtig geïnduceerd te worden door immuuncomplexen. Deze bevinding is relevant voor de pathofysiologische rol van TNF in immuuncomplexziektes zoals rheumatoïde artritis. Op monocysten komen twee verschillende Fc γ receptoren tot expressie, een 72 kDa receptor met hoge affiniteit voor Fc ligand (Fc γ RI), en een 40 kDa receptor met lage affiniteit voor Fc ligand (Fc γ RII). De rol van beide receptoren bij de regulatie van secretie van TNF door monocysten wordt beschreven in hoofdstuk 5. Op onbehandelde monocysten leidt alleen cross-linking van Fc γ RI tot TNF secretie. Echter, na behandeling van de monocysten met proteolytische enzymen of met het deglycosylerende enzym neuraminidase kan TNF secretie ook door cross-linking van Fc γ RII worden geïnduceerd. Dit berust op een toename van de binding van Fc ligand aan Fc γ RII na behandeling van de monocysten met deze enzymen, waardoor effectievere cross-linking van Fc γ RII kan plaatsvinden. Tevens wordt aangetoond dat TNF secretie alleen geïnduceerd kan worden middels Fc-Fc γ receptor interacties, maar niet wanneer cross-linking van Fc γ

receptoren door monoclonale anti-Fc γ receptor antilichamen plaatsvindt. Deze antilichamen binden met het F(ab')₂ deel aan verschillende epitopen van Fc γ receptoren, waarbij cross-linking van Fc γ receptoren plaatsvindt zonder dat TNF secretie wordt geïnduceerd. Deze bevindingen suggereren dat Fc γ RII fungeert als een 'stand-by' receptor, die onder invloed van proteases en neuraminidase, vrijkomend in ontstekingsgebieden, functioneel wordt ten aanzien van de inductie van TNF secretie.

In hoofdstuk 6 wordt het effect bestudeerd van twee verschillende corticosteroiden, prednisolon en budesonide, op de secretie van TNF door monocyten, geïnduceerd door verschillende stimulerende agentia. Beide corticosteroiden remmen dosis-afhankelijk TNF secretie, maar de mate van remming is afhankelijk van het stimulerend agens. Sterke remming treedt op wanneer TNF secretie wordt geïnduceerd door LPS of na cross-linking van Fc γ receptoren. Slechts geringe remming wordt gezien na inductie van TNF secretie door het proteïne kinase C-activerende agens PMA. Intermediaire remming treedt op na inductie van TNF secretie door silica quartz. Tevens wordt aangetoond dat remming van TNF secretie door beide corticosteroiden snel reversibel is. De aanwezigheid van corticosteroiden tijdens de fase van celactivatie is noodzakelijk voor optimale remming van TNF secretie. Deze bevindingen verklaren een van de mechanismen van het immunosuppressieve en anti-inflammatoire effect van corticosteroiden *in vivo*. TNF wordt beschouwd als een cruciale endogene mediator betrokken bij de pathogenese van septische shock. Het sterk remmende effect van corticosteroiden op de door endotoxine geïnduceerde TNF secretie verklaart mogelijk ten dele de beschermende werking van corticosteroiden in experimentele sepsisstudies in proefdiermodellen. Daarnaast draagt remming van de door Fc γ receptoren geïnduceerde TNF secretie bij tot de therapeutische werkzaamheid van corticosteroiden bij immunocomplexziekten zoals reumatoïde arthritis, waarin TNF mogelijk een pathofysiologische rol speelt.

In hoofdstuk 7 wordt aangetoond dat behandeling van acute niertransplantaatrejectie met antithymocyten globuline (ATG) gepaard gaat met een verhoging van de plasma TNF spiegel. De bijwerkingen van deze therapie, zoals koorts, koude rillingen, misselijkheid, braken, dyspnoe e.a., treden op in de periode dat de plasma TNF spiegel verhoogd is. Toediening van de tweede dosis ATG aan de niertransplantatiepatiënten van onze studie gaat slechts met geringe bijwerkingen gepaard, en plasma TNF spiegels zijn dienovereenkomstig laag. Dit suggereert dat TNF, althans ten dele, verantwoordelijk is voor het ontstaan van deze bijwerkingen. Deze conclusie wordt gesteund door bevindingen uit fase I studies van recombinant TNF (rTNF) als antitumor middel in de mens. De bijwerkingen van toediening van rTNF aan mensen gelijken sterk op de bijwerkingen van toediening van anti-T cel antilichamen zoals ATG. *In vitro* experimenten tonen aan dat ATG de secretie van TNF door mononucleaire cellen induceert. Dit wordt niet veroorzaakt door activatie of lysis van T lymphocyten. Mogelijk wordt TNF in deze experimenten gesecreteerd door monocyten na opsonisatie van, met ATG gecoat, lymphocyten via een door Fc receptoren gemedieerd proces.

In hoofdstuk 8 wordt tenslotte aangetoond dat bij septische patiënten in een van de vier gevallen verhoogde TNF spiegels in het plasma aantoonbaar zijn. De mortaliteit van patiënten met aantoonbare plasma TNF spiegels blijkt significant hoger te zijn dan die van septische patiënten zonder aantoonbare TNF spiegels. Het verschil in morta-

liteit lijkt niet verklaard te kunnen worden door verschillen in ernst van de sepsis of ernst van het multi-orgaan falen (MOF), aangezien de parameters gebruikt om de ernst van sepsis of MOF te schatten, niet verschillen tussen beide groepen patiënten. Talrijke dierexperimentele studies bevatten aanwijzingen dat TNF een belangrijke pathogenetische betekenis heeft voor het ontstaan van shock en MOF tijdens sepsis. Twee klinische studies hebben een verband aangetoond tussen de aanwezigheid van circulerend TNF en mortaliteit bij patiënten met meningococcensepsis. Onze studie bevestigt deze bevindingen voor een populatie septische patiënten met een breed scala aan onderliggende ziektes.

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Tenslotte Lisette, 42 km lang liep jij met mij mee. Ook bij een promovendus geldt: 'cherchez la femme'.

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 9 augustus 1958 te Kerkrade. Hij behaalde in 1976 het gymnasium β diploma aan het gymnasium Rolduc in zijn geboorteplaats. Van 1976 tot 1982 studeerde hij geneeskunde aan de Rijksuniversiteit Limburg te Maastricht, alwaar hij in 1982 het artsdiploma behaalde. Na werkzaamheden als poortarts in het St. Jans Gasthuis te Weert en als assistent-chirurg in het Havenziekenhuis te Rotterdam, begon hij in 1985 met de opleiding chirurgie in het Academisch Ziekenhuis Maastricht (Prof. Dr. J.M. Greep en Prof. Dr. G. Kootstra). Van juli 1986 tot juli 1988 verrichte hij in het biomedisch laboratorium van de vakgroep Algemene Heelkunde de in dit proefschrift beschreven wetenschappelijke studie.