TUMOR NECROSIS FACTOR-ALPHA

ANTITUMOR AND IMMUNOMODULATORY EFFECTS

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TUMOR NECROSE FAKTOR-ALFA
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De vreze des Heren is het begin der wijsheid

Psalm 111 vers 10[A]

Ter nagedachtenis aan mijn vader
Aan mijn moeder
Aan Margrèt

CONTENTS

GENERAL INTRODUCTION

All animals possess an immune system in order to protect and preserve their integrity. The essential task of the immune system is the discrimination between "self" and "non-self" and the generation of an immune response to rid the organism of "non-self" elements. In recent years knowledge of the immune system has rapidly expanded. It is becoming increasingly clear that, in order to fulfill its complex tasks, the immune system uses an ingenious communication system. Communication between immune cells is performed in various ways, one of them by the agency of soluble mediators, generally termed cytokines. From a clinical point of view it is of great interest to analyse the effects of these cytokines. Manipulation of their release or actions might offer new perspectives for clinical entities in which stimulation or suppression of the immune system is essential, such as in cancer immunotherapy and in organ transplantation.

Immunotherapy is, besides surgery, radiotherapy, and chemotherapy, the fourth treatment modality of cancer. It is based on the hypothesis that developing tumors can be antigenically different from normal tissues, and consequently can be recognized as "non-self". Indeed it has been demonstrated that some tumors are antigenically different from normal tissue, either by re-expressing embryonic antigens or differentiation antigens or by altered expression of "normal" antigens. Therefore cancer immunotherapy might be a successful treatment modality. Immunotherapy can be performed in several ways. It can be accomplished by adoptive transfer of immunocompetent cells, either being lymphokine-activated T-cells or lymphokine-activated killer-cells (LAK-cells), by transfer of antibodies conjugated with a toxine or by administration of excess amounts of cytokines in order to stimulate the host's own immune system to recognize the tumor and respond to it.
In the first part of this thesis we examined the possibilities of using the cytokine tumor necrosis factor-alpha (TNFα) for the latter aproach. Although multiple studies have been performed regarding the antitumor action of TNFα, it is still not clear whether TNFα has sufficient potential to be used as immunotherapeutic agent in vivo. In this part of this thesis an answer to this important question is given.

Whereas cancer immunotherapy might benefit from immunostimulation with cytokines such as TNFα, immunosuppression through the inhibition of the release or activities of cytokines might be to the advantage of recipients of organ grafts. Rejection of a donor organ by a recipient is known to be an immunological process in which various cells of the immune system work together. Immune cells recognize the graft, either directly or indirectly, as being "non-self", and according to their task, they generate an immune response to rid the recipient of the "non-self" graft. Cytokines play a crucial role in this proces. Inhibition of cytokine production via cyclosporin-A delays or prevents graft rejection. The role of the cytokine interleukin-2 has been well established in this proces. Less attention has been paid to the involvement of TNFα. Maury and Teppo (1987) demonstrated that TNFα is present in the serum of patients with rejecting kidney grafts. This, however, did not prove that TNFα is actually involved in the process of graft rejection. Therefore experiments were performed in rats to assess the significance of TNFα with regard to graft rejection. This was done either by administration of recombinant TNFα to rats with organ grafts or by blocking of TNFα activity in such rats by means of anti-TNFα antibodies. These experiments are described in the second part of this thesis.

Each part of the thesis ends with the conclusions that can be drawn from the respective experimental results.

PART 1

CHAPTER 1

MOLECULAR AND BIOLOGICAL CHARACTERISTICS OF TUMOR NECROSIS FACTOR-ALPHA

This chapter is an adapted version of the article

TNF: A brief review with emphasis on its antitumor activity
by M. Scheringa and R.L. Marquet

Biotherapy 2: 275-281, 1990

INTRODUCTION

The tumor necrotic effects of bacterial products have been known for a long time (reviewed by Ruff & Gifford 1981, Table 1.1). More than a century ago Coley (1891) observed that spontaneous regression occurred in some cancer patients who had concurrent bacterial infection. This observation led to the discovery that hemorrhagic necrosis of certain mouse tumors could be reproducibly obtained by injection of filtrates from cultures of gram-negative bacteria. Shear and coworkers (1943) identified the active principle as a polysaccharide. This polysaccharide turned out to be a major constituent of the cell wall of gram-negative bacteria and is now known as lipopolysaccharide or endotoxin. The fact that endotoxin did not kill tumor cells in culture indicated that its action must be indirect, and lent credence to Alkire's conclusion that hemorrhagic necrosis might be secondary to endotoxin-induced hypotension leading to circulatory stasis and ischemia in the tumor (Algire et al. 1952). The idea that tumor necrosis was a secondary reaction was confirmed in 1975 by the finding of a endotoxin-induced serum factor which caused necrosis of various tumors in mice (Carswell et al. 1975). Activated macrophages were suggested as the cellular source of this serum factor which was named Tumor Necrosis Factor (TNF, later TNFα). In search of cytokines that inhibit tumor cell growth, TNF was first isolated and purified by Aggarwal et al. in 1984 (published in 1985) and its cDNA was isolated by Pennica et al. in the same year. The isolation of TNF was a by-product of the isolation of another tumor cell growth inhibiting cytokine, previously termed Lymphotoxin, but now renamed TNFβ.

Since the availability of recombinant TNFα, knowledge of TNFα has rapidly expanded. The next section gives a short review of the pleiotropic effects of TNFα as known currently, with special emphasis on its antitumor activity.

Table 1.1 History of tumor necrosis factor-α

Date	Event	Reference
1891	Cancer patients were treated with mixtures of killed Streptococcus pyogenes and Serratia marcescens preparations.	Coley 1891
1931	Demonstration of hemorrhagic necrosis of animal tumors after injection of bacterial filtrates.	Gratia et al. 1931
1943	Bacterial agent that induces hemorrhagic necrosis identified as a polysaccharide.	Shear et al. 1943
1952	Proposal that endotoxin-induced hypotension leads to vascular collapse and hemorrhagic tumor necrosis.	Algire et al. 1952
1968	Lymphotoxin (TNFβ) produced by antigen-stimulated or mitogen-stimulated lymphocytes shown to cause target cell lysis.	Ruddle et al. 1968 Granger et al. 1968
1975	Factor capable of inducing tumor necrosis and distinct from endotoxin characterized from mouse serum.	Carswell et al. 1975
1984	Human lymphotoxin cDNA cloned.	Gray et al. 1984
1984	Human tumor necrosis factor-α cDNA cloned.	Pennica et al. 1984

TNFα MOLECULE AND GENE

TNFα and TNFβ are structurally related (Pennica et al. 1984), and their genes are located on the same chromosome. In man the genes are located on the short arm of chromosome 6 in the region of major histocompatibility genes (Spies et al. 1986, Mueller et al. 1987). Polymorphism in the gene for TNFα has not been reported. The gene for TNFα is approximately 3 kilobases in size and contains 3 introns. The TNFα

gene is highly conserved in mammalian species resulting in a 80% homology at the amino acid level between human and murine TNFα (Marmenout et al. 1985). Mature human TNFα is secreted after cleavage of a 76-residue peptide from the aminoterminus of the prohormone and contains a single, intramolecular disulfide bridge (Aggarwal et al. 1985). Analytical ultracentrifugation, cross-linking (Smith et al. 1987), small angle x-ray scattering (Lewit-Bentley et al. 1988, Eck et al. 1989), and gel-electrophoretic studies (Eck et al. 1988) have shown that TNFα is a trimer in solution. The monomer of human TNFα is a non-glycosylated protein with a molecular weight of 17 kilodalton and consists of 157 amino acid residues (Pennica et al. 1985).

SOURCES AND REGULATORS OF TNFα

TNFα was first described as a cytokine produced by activated monocytes, but we know now that it can be produced by a variety of cell types such as lymphocytes (Sung et al. 1988)), natural killer cells (Pistoia et al. 1989, Moretta et al. 1990), Kupffer cells (Roh et al. 1990), mast cells (Galli et al. 1989), and astrocytes (Chung et al. 1990). Besides endotoxin, the classical inducer of TNFα, various other agents have been shown to induce TNFα production. They include interferons (Rutenfranz et al. 1990), interleukins (Delwel et al. 1990, Kasid et al. 1990), viruses (Nain et al. 1990), parasites (Taverne et al. 1990), and tumor cells (Hasday et al. 1990). Inhibition of TNFα production can be achieved by cyclosporin (Goldin et al. 1989, Nguyen et al. 1990), lipoxygenase inhibitors (Schade et al. 1989, Dubois et al. 1989), some interleukins (Kasid et al. 1990), and various other agents.

TNFα RECEPTOR

TNFα exerts its cellular responses by binding to high-affinity cell-surface receptors, present on most somatic cells (Kull et al. 1985, Scheurich et al. 1986). Two types of

TNFα receptors, termed type A and B, have been demonstrated (Hohmann et al. 1989, Brockhaus et al. 1990). They were shown to be proteins with apparent molecular masses of 75 and 55 kDa, respectively. Cell lines have been shown to express very different ratios of the types A and B receptors. Furthermore it was found that the expression of A and B receptors can be regulated independently (Hohmann et al. 1990).

TNFα and TNFβ bind to the same receptors. This was first suggested by Aggarwal et al. (1985) who found that the binding of ^{125}I-TNFα to human cervical carcinoma cell line ME-180 could be competitively displaced by equivalent amounts of either unlabeled TNFα or unlabeled TNFβ. Recently this finding has been confirmed by other experiments (Stauber et al. 1989, Hohmann et al. 1989, 1990). Even though they share a common receptor, TNFα and TNFβ do not share the same biological response in several cell types (Locksley et al. 1987), suggesting differences in the post receptor events for these cytokines. The post receptor events of TNFα and TNFβ have still not been fully elucidated.

Recently TNFα inhibitors have been demonstrated in urine of febrile patients (Seckinger et al. 1988). These inhibitors have now been purified and they appear to be similar to parts of the TNFα receptor (Seckinger et al. 1990). Therefore it seems that there are also soluble TNFα receptors as already described for other cytokines (Cornaby et al. 1989, Noronha et al. 1990).

BIOLOGICAL ACTIVITIES OF TNFα

Although originally described as a cytokine with tumor necrotizing ability, TNFα has now been recognized as having pleiotropic activities (Table 1.2), both in vitro and in vivo. Each of these activities is briefly described in the next section.

Table 1.2 Pleiotropic effects of TNFα

- Mediator of endotoxic shock
- Modulator of endothelial cell functions
- Cell growth promoting activity
- Antiviral activity
- Antiparasitic activity
- Antitumor activity
- Immunomodulatory activities

TNFα AND ENDOTOXIC SHOCK

The role of TNFα in septic shock and cachexia (Reviewed by Tracey et al. 1988, and Fong et al. 1990) became clear when it was recognized that TNFα and the protein cachectin were one and the same molecule (Caput et al. 1986). Cachectin was discovered during studies of rabbits infected with Trypanosoma brucei in an experimental model of cachexia associated with extreme weight loss, depletion of lean body mass, and hypertriglyceremia. It was found that serum levels of triglycerides rise because of a systemic suppression of lipoprotein lipase (LPL, Rouzer et al. 1980). Subsequent work showed that mice injected with endotoxin produce a serum factor that causes LPL suppression (Kawakami et al. 1981). This factor was isolated from the supernatant of endotoxin-induced murine macrophages by Beutler et al. (1985), and the name cachectin was given because of its suspected role as a mediator of cachexia. Cachectin's NH_2-terminal amino acid sequence (Beutler et al. 1985) and receptor binding specificity were strongly homologous to those of TNFα. When the cDNA for both the cachectin and TNFα genes became available, it was confirmed that both molecules were identical (Caput et al. 1986).

When highly purified recombinant TNFα is administered intravenously (i.v.) in

animals, the resultant state of tissue injury, acidosis, and shock is pathologically and physiologically indistinguishable from septic shock. Fever, falling blood pressure, myocardial suppression, dehydration, and acute renal failure are followed rapidly by abrupt respiratory arrest. Elevated serum levels of lactate, catecholamines, and glucagon are observed and reflect a state of inadequate tissue perfusion and net catabolism. Animals succumbing to TNFα-induced shock show evidence of hemorrhagic necrosis in kidney, lung and intestine (Tracey et al. 1986, Remick et al. 1987, Marquet et al. 1987).

In animals as well as in man elevated serum-TNFα levels have been reported during septic periods. Moreover prophylactic and therapeutic effects of monoclonal antibodies to TNFα in experimental gram-negative shock have been reported. It has also been claimed that monoclonal antibodies to the endotoxin core can protect mice from gram-negative sepsis without inhibiting the endotoxin-induced TNFα production, thus suggesting that septic shock is also due to TNFα independent mechanisms (Silva et al. 1990). These could be the induction of other endotoxin-induced cytokines (Fong et al. 1989).

TNFα AS A MODULATOR OF ENDOTHELIAL CELL FUNCTIONS

The effects of TNFα on endothelium have been thoroughly examined (reviewed by Pober et al. 1987). Various biological activities of TNFα are mediated in part by modulation of endothelial cells.

Effect of TNFα on coagulation: Normal endothelium is in an anticoagulant state. Upon stimulation with TNFα the endothelial cells confer into a procoagulant state by several ways. TNFα induces endothelial cells to synthesize tissue factor, which initiates blood coagulation (Nawrothe et al. 1986, Bevilacqua et al. 1986); thrombomodulin activity, an antithrombotic component of endothelial cells, is reduced upon TNFα stimulation (Nawroth et al. 1986), and the expression of plasminogen activator inhibitors is increased (Van den Berg et al. 1988, Schleef et al. 1988). Phorbol myristate acetate stimulation of endothelial cells mimics most, but not all of

these effects of TNFα, suggesting that TNFα exerts these effects not only through stimulation of protein kinase C (Scarpati et al. 1989). All these actions of TNFα can lead to fibrin deposition and clot formation, effects reported to be for example essential in the anti-tumor effects of TNFα (Shimomura et al. 1988).

Effect of TNFα on morphology and phenotype of endothelial cells: Activated endothelial cells present in lymphoid organs and at sites of chronic inflammation often develop a tall, columnar or cuboidal morphology, a large ovoid nucleus with dispersed chromatin, prominent nucleoli, and increased numbers of intracellular organelles (Smith et al. 1970, Dvorak et al. 1976, Nightingale et al. 1978, Freemont 1983, Freemont 1985, Iguchi 1986). Dumonde et al. (1982) found that this activated state of endothelium could be induced by crude cytokine preparations. After purification it became clear that various cytokines, among which TNFα, could activate endothelium (Stolpen et al. 1986, Montesano et al. 1985, Sato et al. 1986, Cavender et al. 1989).

Another feature of activated endothelial cells is the increased binding capacity to leucocytes. TNFα induces the expression of specific binding receptors on endothelium like endothelial leukocyte adhesion molecule (ELAM)-1 (Bevilacqua et al. 1987, Munro et al. 1989), and intercellular adhesion molecule (ICAM)-1, Munro et al. 1989). TNFα stimulates thereby the actual migration of leucocytes through the endothelial lining (Moser et al. 1989).

ROLE OF TNFα AS GROWTH FACTOR

Apart from being cytolytic to tumor cells, TNFα stimulates growth of certain normal cells like fibroblasts, thymocytes, B-lymphocyters, and hepatocytes (Sugarman et al. 1985, Ranges et al. 1987, Cordingley et al. 1988, Beyer et al. 1990). TNFα also promotes angiogenesis and liver regeneration after partial hepatectomy, suggesting a physiological role of TNFα during wound healing (Frater-Schroeder et al. 1987, Leibovich et al. 1987, Beyer et al. 1990).

ANTIVIRAL AND ANTIPARASITIC PROPERTIES OF TNFα

One of the physiological characteristics of TNFα might be its protective role during various viral, fungal, parasitic, and bacterial infections. It has been demonstrated to exhibit antiviral effects against both DNA and RNA viruses (Wong et al. 1986, Mestan et al. 1986, Vilcek et al. 1988). It plays a protective role in mice infected with the protozoa parasite Leishmania major (Titus et al. 1989), and it activates macrophages to kill Trypanosoma cruzi (Wirth et al. 1988). Anti-malaria activity of TNFα has also been reported (Shaffer et al. 1991).
Growth of several bacteria, such as Listeria monocytogenes (Havell 1987) and Bacillus Calmette Guérin (Kindler et al. 1989), is inhibited by TNFα. Anti-fungal activities of TNFα have been reported for example against Candida albicans (Djeu et al. 1986). These effects of TNFα are probably indirectly mediated by the immune system.

IMMUNOMODULATORY ACTIVITIES OF TNFα

TNFα modulates various immunological reactions, either alone or in concert with other cytokines (see chapter 6). Macrophage cytotoxicity is enhanced by TNFα, especially when TNFα is combined with LPS or interferon gamma (Hori et al. 1989). Polymorphonuclear granulocytes lyse tumor cells better when incubated with TNFα (Shan 1988) and they adhere better to endothelium (Gamble et al. 1985). T-cells grow faster when stimulated in the presence of TNFα (Yokota et al. 1988), and TNFα and interleukin 2 act synergistically in LAK-cell generation (Owen-Schaub et al. 1988). Because of its widely ranging immunological activities, TNFα has been described as important mediator of various immunological abnormalities, such as autoimmune diseases (Jacob et al. 1988). TNFα is also involved in host-versus-graft and graft-versus-host reactions (Maury et al. 1987, Piquet et al. 1987). The severity of these reactions can be diminished by blocking of TNFα activity (see chapters 7, 8, Imagawa et al. 1990, Shalaby et al. 1989).

TNFα AS ANTITUMOR AGENT

In vitro: It was first reported that TNFα had cytostatic and/or cytolytic effects against various transformed cell lines (Nio et al. 1988, Fransen et al. 1986), while it had no or growth promoting effects on normal cell lines (Fransen et al. 1986, Sugarman et al. 1985). In general this still holds, although it has been demonstrated that TNFα in combination with IFNγ can be cytolytic to normal rat embryo fibroblast-like cells (Suffys et al. 1989), and that it stimulates the growth of some B-cell leukemias (Digel et al. 1989).

The mechanism of in vitro cytotoxicity of TNFα is still not fully elucidated. TNFα treatment of sensitive cells leads to membrane perturbations and DNA fragmentation (Rubin et al. 1988). In these processes the role of oxygen radicals seems to be important. The addition of free radical scavengers can suppress the lytic effects of TNFα (Matthews et al. 1987); in cells resistent to TNFα the levels of manganous superoxide dismutase are elevated (Wong et al. 1988), and cells with inadequate radical buffering capacity are sensitive to TNFα (Zimmerman et al. 1989). The suspected role of free radical scavengers may well be in agreement with the former finding that protein inhibitors increase the susceptibility of cells to TNFα (Darzynkiewitcz et al. 1987). Protein inhibitors prevent the production of O2 scavengers in the cell which would make them more susceptible to TNFα.

In vivo: TNFα given intravenously (i.v.) or intralesionally (i.l.) has tumor growth inhibiting and/or tumor necrotizing ability (see chapter 2, Talmadge et al. 1987, Carswell et al. 1975, Balkwill et al. 1986), sometimes leading to complete cures (Haranaka et al. 1984). In various animal models it has been shown that i.l. administration is more effective than other ways of administration (see chapter 2, Haranaka et al. 1984). This probably reflects the fact that local administration allows for higher doses of TNFα than systemic administration. High doses given by continuous infusion are not advantageous to repeated bolus injections (see chapter 2). In general it appears that TNFα, in single agent therapy, rarely results in complete cures.

TNFα has been shown to have synergistic antitumor effects with interferons, both in

vitro and in vivo (Van der Heyden et al. 1986, Schiller et al. 1987, 1990, Marquet el al 1987). This synergy was explained by the fact that interferon induces the expression of TNFα receptors on the cell membrane of the tumor cells (Ruggiero et al. 1986). Synergy of TNFα with various other agents or treatments has been reported, like TNFα with hyperthermia (Klostergaard et al. 1989, Iizumi et al. 1989), and TNFα with chemotherapeutics (Das et al. 1989, Schiller et al. 1990). Fairly new is the combination of induced endogenous TNFα with exogenous TNFα, the so-called endogenous-exogenous therapy (EET, see chapter 3, Inugawa et al. 1988). This therapy has been reported to be very effective in animals and in man (Inugawa et al. 1988).

Mechanism of action in vivo: Various tumor cells are insensitive to TNFα in vitro but sensitive to TNFα in vivo (Havell et al. 1988). This discrepancy has led to the hypothesis that TNFα exerts its effect in vivo through one or more host factors. The immune system seems to be important because TNFα has better effects in heterozygous (nu/+) littermates than in homozygous (nu/nu) nude mice (Haranaka et al. 1984). When normal mice are pretreated with L3T4 monoclonal antibodies TNFα looses its antitumor effect (Palladino et al. 1988), and when rats are treated with TNFα when they are immunosuppressed, TNFα looses its effect as well (see chapter 2). Besides the immune system, the effects of TNFα on tumor vessels seems to play a role. TNFα has better effects in some models in vascularized than in non-vascularized tumors, and it has been reported that the clotting, seen after TNFα treatment is essential for TNFα's antitumor activity (Shimomura et al. 1988). This led North and Havell (1988) to suggest that for a complete cure after TNFα treatment the effects of TNFα on vessels as well as the immunomodulatory effects of TNFα are important; the former leading to tumor necrosis and the latter leading to complete eradication of the tumor.

Clinical results: So far TNFα has only been tested in phase 1 or combined phase1/2 studies (see chapter 4). Systemic toxicities which have been described, consisted of fever, preceded by chills/rigors, headaches, fatigue, nausea/vomiting, hypotension, and sometimes cardiac and hepatic toxicities (Blick et al. 1987, Selby et al. 1987, Chapman et al. 1987, Padavic et al. 1988, Conkling et al. 1988, Creagan et al. 1988,

Feinberg et al. 1988, Bartsch et al. 1989, Creavan et al. 1989, Pfreundschuh et al. 1989). Neurologic toxicities were scarce. Chills, rigors and fever were dose-independent. Toxicity, especially fever, could be ameliorated by prophylactic treatment with non-steroid anti-inflammatory drugs (NSAID) such as meperidine or ibuprofen, whereas rigors did not respond to NSAID. Headaches responded well to treatment with acetaminophen. Hypotension is sometimes preceded by a rise in tension shortly after drug administration; volume substitution is only necessary at high doses of TNFα. Cardiac toxicities consist of tachycardia which is never dose limiting. Hematologic toxicities consist of a drop in platelet count, leukopenia and sometimes a slight granulocytosis and thrombocytopenia. NK-cells are diminished shortly after TNFα administration. All toxicities mentioned have been found to be reversible within 1 or 2 days. TNFα has been administered through the i.v. route, sometimes combined with subcutaneous (s.c.) or intra-muscular administration (i.m.) and through the i.l. route. Reported maximum tolerated doses (MTD) for the various routes did not differ much and were for the i.v. route between $3x10^5$ U/m^2/day and $6x10^6$ U/m^2/day and for the i.l. route between $9x10^5$ U/m^2 and $1.2x10^6$ U/m^2. The reported MTD for the i.m. and s.c. route of administration were $8x10^6$/m^2 and $5x10^6$/m^2, respectively. The overall responses reported so far were for the i.v. route (n=7 trials) 12% and for the i.l. route (n=2 trials) 50%. Thus both in patients and in animals i.l. administration seems preferable in terms of efficacy. Combination treatments with other cytokines in the clinic have as yet not been fully explored but the first data show that TNFα acts indeed synergistically with interferons (Demetri et al. 1989).

CONCLUSION

TNFα is a highly active cytokine both in vitro and in vivo. It displays both desirable and undesirable activities. In order to develop TNFα as a therapeutic product, one must find a way to down modulate its undesirable toxicities and upregulate its efficacious potency by perhaps using it in combination with other cytokines such as interferons.

CHAPTER 2

ANTITUMOR EFFECT OF TNFα; A STUDY OF THE EFFICACY AND MECHANISM OF ACTION OF TNFα IN A RAT COLON CARCINOMA MODEL

This chapter is a modified version of the article

Anti-tumor effect of recombinant murine TNF-alpha (rMuTNFα) given by continuous i.v. infusion as compared to repeated i.v. injections in a rat liver metastasis model

by M. Scheringa, A. Keizer, J. Jeekel, and R. L. Marquet

Int. J. Cancer 43: 905-909, 1989

SUMMARY

In the present study we examined the antitumor efficacy of TNFα in a rat model using colon carcinoma CC531. In addition we performed a series of experiments in order to unravel its mechanism of antitumor activity. Coloncancer CC531 is chemically induced in WAG rats.
A problem in using TNFα therapeutically is its toxicity. In rats a single intravenous (i.v.) injection of 40 μg/kg of rMuTNFα is lethal. When given by continuous i.v. infusion, doses up to 200 μg/kg/day are well tolerated. The higher levels that can be achieved by continuous infusion do, however, not result in higher anti-tumor efficacy, as was shown in an artificial liver metastases model. Local administration of TNFα within a tumor also allows for higher doses to be administered and it was demonstrated that this way of administration indeed has more anti-tumor potential.
Tumor CC531 was shown to be highly resistent to TNFα's actions in vitro, suggesting that the in vivo effects of TNFα are mediated by a host factor. This turned out to be the immune system as was demonstrated by the observation that TNFα looses its antitumor effect when given to rats that are pretreated with an immunosuppressive agent (cyclosporin-A). Pretreatment of rats with heparin did not influence TNFα's antitumor actions, indicating that clotting is of no importance for TNFα's effect in our rat model.

INTRODUCTION

TNFα, a monocyte/macrophage derived protein was first described as a mediator of lipopolysaccharide-induced hemorrhagic necrosis of certain sarcomas in mice, and as a molecule with cytostatic/cytolytic activity for tumor cells in vitro (Carswell et al. 1975). Although TNFα can cause hemorrhagic necrosis and regression or even total cure of several murine tumors and human tumors transplanted in mice (Balkwill et al. 1986, Chung et al. 1987, Agah et al. 1988), its therapeutic use is hampered by its toxicity. TNFα causes, for example, severe wasting, diarrhea, ischemic and hemorrhagic lesions of the gastrointestinal tract and suppresses lipoprotein lipase activity (Marquet et al. 1987, Beutler et al. 1987). In 1985, Beutler et al. noted that cachectin, a molecule known to cause cachexia in infectious and neoplastic disease,

possessed an identical spectrum of bioactivities as TNFα and that the amino terminal sequence of mouse cachectin was strongly homologous to that of TNFα, suggesting that they were in fact the same molecule, as was confirmed by genetic sequence analysis (Caput et al. 1986). Bearing this in mind TNFα should be used even more carefully.

The following experiments were performed to examine the efficacy of TNFα treatment of a colon carcinoma, and to examine whether administration of TNFα via continuous infusion might be better than administration via bolus injections, both in terms of toxicity and of efficacy. CC531, a chemically induced colon adenocarcinoma, transplantable in WAG-rats, was used. At first the sensitivity of CC531 for TNFα treatment was tested in vitro using various doses of TNFα. Then the sensitivity of CC531 was examined in vivo. The tumor was implanted under the renal capsule, subcutaneously or injected via the portal vein into the liver. TNFα was administered either systemically or locally at various doses. In addition a series of experiments were performed in order to unravel the mechanism of antitumor action of TNFα.

MATERIALS AND METHODS

Animals:

Male rats of the inbred WAG ($RT1^u$) strain were obtained from Harlan-CPB (Austerlitz, the Netherlands). The animals were bred under specific pathogen-free conditions and were 10 to 14 weeks old when used. The animals were kept under standard laboratory conditions (12 hours light/12 hours dark) and were fed a standard laboratory diet (Hope Farms, Woerden, The Netherlands).

Tumor:

A 1,2 dimethylhydrazine-induced, moderately differentiated colon adenocarcinoma (CC531), transplantable in syngeneic WAG rats was used (Marquet et al. 1984). The tumor was maintained in vitro in RPMI 1640 medium supplemented with 5% fetal calf serum (virus and mycoplasma screened), 1% penicillin (5000 U/ml), 1%

streptomycin (5000 U/ml) and 1% L-glutamine (200 mM). All supplementaries were obtained from Gibco (UK). Before usage cells were trypsinized (5 min, 37°C), centrifuged (5 min, 700xg), resuspended in RPMI 1640 and counted. Viability was measured with trypan-blue (0.3% in a 0.9% NaCl-solution). Viability always exceeded 95%. The tumor is weakly immunogenic as determined by the immunization-challenge method of Prehn and Main (1957, Marquet et al. 1987).

In vitro testing of tumor cell line for response to TNFα:

Tumor cells were seeded at $1x10^4$ cells per well in flat-bottomed 96-well microtiter plates (Costar, USA) in a final volume of 0.2 ml medium per well (see "Tumor"), and incubated at 37°C in 5% CO_2 for 48 h in the presence of different concentrations of rMuTNFα(F). Concentrations rMuTNFα used were 0 U/ml (0 ng/ml), 50 U/ml (0.17 ng/ml), 100 U/ml (0.34 ng/ml), 1000 U/ml (3.4 ng/ml) and 5000 U/ml (17.2 ng/ml). Growth of tumor cells was measured using an MTT-assay (Romijn et al. 1988); after 48 h incubation, 30 μl of a 5 mg/ml solution of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide] (MTT, obtained from Sigma, St. Louis, MO) in phosphate-buffered saline was added. After 3.5 hours of incubation with MTT at 37°C, the supernatant was carefully sucked off, and 0.1 ml dimethylsulfoxide was added to each well. The plates were placed in a microplate shaker for 1 min and the absorbance at 515 nm was read using a Flow Titertek Multiscan plate reader. Eight replicate wells were used for each group to be tested. Wells containing medium but no cells served as blanks. Statistical significance between different groups was assessed using analysis of variance followed by a Student-Newman-Keuls test.

Sub-Renal Capsule Assay (SRCA):

Recipient animals were anesthetized with ether, a midline incision was made and both kidneys were exteriorized. A small knick was made in the capsule and a tumor fragment of about 10 mg (starting weight of all individual tumors was measured) was implanted and pushed up to the upper kidney pole. After 1 or 2 weeks the animals were sacrificed, the tumors enucleated, and subsequently weighed. Statistical significance between groups was assessed using Wilcoxon's Rank Sum test.

Subcutaneously grown tumor:

Recipient animals were anesthetized with ether, a small incision was made in the right flank of the animals, and a tumor piece of about 10 mg was placed subcutaneously. Tumor growth was assessed by measuring with callipers the two largest perpendicular diameters at regular time intervals. The average diameter was taken as measure of tumor size. When the tumordiameters were between 1.0 cm and 1.5 cm treatment was started. Statistical significance between the different groups was assessed using Wilcoxon's Rank Sum test.

Induction and measurement of liver metastases:

Tumor cells were injected via the portal vein into the liver ($5x10^5$ cells of the in vitro cell line in 0.5 ml HBSS, day 0). Tumor growth was measured by macroscopic counting of the number of liver metastases at day 28. For that reason animals were killed and a median laparotomy was performed. When more than 60 metastases were present, individual metastases were no longer countable.

Therefore a scoringssystem was introduced:

number of liver metastases:	score:
0	0
1-10	2
11-30	3
30-60	4
>60	5
>>60	6

A score of 5 was given when more than 60 metastases were present and still a great part of the liver was without any tumor. A score of 6 was given when more than 60 metastases were present and hardly any or no normal liver tissue was left. The score is not equidistant because having no liver metastases at all was considered very important. Statistical significance between different groups was assessed using the Wilcoxon's Rank Sum test for the raw data and the Chi-square test for means for the introduced scoringsystem.

Continuous infusion:

Under ether anesthesia, rats were cannulated via the jugular vein (canules: Silastic, ID 0.025 in., OD 0.047 in. obtained from Dow Corning, Michigan, USA) and subsequently connected to an infusion pump (H.R.Flow Inducer, MHRE 7, obtained from Watson-Marlow Ltd., UK). Rats could move freely because of the use of a swivelling system. After 4 days of infusion rats were disconnected from the pump and placed in their normal cages.

TNFα:

Two samples of recombinant murine TNFα (rMuTNFα) were used. One of them was kindly provided by dr. Fiers (Roche Research Gent, Belgium), referred to as rMuTNFα(F). This preparation used was over 99% pure, containing $2.9x10^8$ U/mg protein and less than 4 ng endotoxin per mg protein. The other was kindly provided by Knoll/BASF (Ludwigshafen), referred to as rMuTNFα(B). This preparation was also over 99% pure, containing $1.4x10^7$ U/mg protein and less than 1.5 ng endotoxin per mg protein (Limulus test). 4 μg/kg of the TNFα provided by dr. Fiers was equivalent to 32 μg/kg of the TNFα provided by Knoll/BASF as measured in terms of efficacy in an SRCA. Details of rMuTNFα production have been published (Fransen et al. 1985).

Luconyl staining:

Luconyl blue (no 708, BASF, FRG), a dye consisting of macromolecules and unable to pass the vascular endothelial lining, was used to visualize the vessels within the tumor. It was kindly provided by dr. Reinhold (TNO, Rijswijk, The Netherlands). The dye was diluted 4:6 in PBS and injected i.v. at a dose of 1.5 ml/rat. Ten minutes after injection of the dye, the animals were sacrificed and histological preparations of the tumor were made. All luconyl-stained vessels within a tumor were counted under a light microscope and the tumor area was assessed. The number of vessels per tumor area was calculated.

Immunosuppression:

Commercially available Cyclosporin A (CsA, Sandoz, Switzerland) was dissolved in olive oil and administered i.m. It was administered at a dose of 50 mg/kg at days -2, -1, and 0 during the experiments in the SRCA model and at a dose of 15 mg/kg at days -2, -1, and 0 and from day 0 three times weekly until the end of the experiment in the s.c. model.

Heparin:

Natrium heparin was obtained from Organon (Boxtel, The Netherlands). To investigate the importance of clotting, animals were pretreated with 75 IU of heparin per animal prior to TNFα administration.

Experimental design:

To examine the toxicity of rMuTNFα(F) given by continuous infusion, rats received 20, 40, 80, 200 or 400 μg/kg/day dissolved in 10 ml HBSS. The animals were observed for signs of toxicity (general appearance, development of diarrhea) and histology of the colon and ileum was taken and examined.

The effects of TNFα on growth of tumor CC531 was assessed in various models. In experiment 1 the antitumor effect of rMuTNFα(B) was measured in a SRCA. The tumors were implanted at day 0, wereafter treatment was started. Control animals received 1 ml HBSS on days 0, 2, and 4. Experimental animals received either 16 μg/kg or 32 μg/kg of rMuTNFα(B) on days 0, 2, and 4. At day 7 the tumors were enucleated and weighed.

In experiment 2 the effect of rMuTNFα(F) on the appearance of livermetastases of tumor CC531 was measured. Animals were treated either early or late. TNFα was administered as repeated bolus injections or as continuous infusion at various concentrations. Experiment 2[a]: Administration by repeated injections: Control animals received 1 ml HBSS i.v. on days 0, 2, and 4 (early treatment) or on days 14, 16, and 18 (late treatment). Experimental animals received 4 μg/kg of rMuTNFα(F) on days 0, 2, and 4 or on days 0 and 7 (early treatment) or on days 14, 16, and 18 or on days 14 and 21 (late treatment). Animals were sacrificed at day 28 and the number of liver

metastases was counted. Experiment 2[b]: Administration by continuous i.v. infusion: Control animals received 10 ml HBSS/day during days 0-4 (early treatment) or during days 14-18 (late treatment). Experimental animals received 4 μg/kg/day during days 0-4 or during days 14-18. One group of animals received a sublethal dose of rMuTNFα(F) (200 μg/kg/day) during days 14-18. At day 28 the animals were sacrificed and the number of liver metastases was counted.

Experiment 3: Tumor CC531 was s.c. implanted and treatment started when the tumors were between 10 and 15 mm in diameter. Control animals received 0.1 ml HBSS intralesionally (i.l.) at days 0, 5, 10, and 15. Experimental animals received either 64 μg/kg of rMuTNFα(B) or 128 μg/kg of rMuTNFα(B) i.l. At days 0, 5, 10, 15, 20, and 25 the tumor diameter was measured.

Experiment 4: The importance of the immunestatus of the animals for the antitumor effect of TNFα was assessed both in a SRCA and in the s.c. model. Experiment 4[a]: Tumor CC531 was implanted under the renal capsule. Control animals received 1 ml HBSS ondays 0, 2, and 4. Control/CsA animals received 50 mg/kg CsA on days -2, -1 and 0 and 1 ml HBSS on days 0, 2, and 4. TNF-treated animals received 4 μg/kg of rMuTNFα(F) on days 0, 2, and 4. TNF/CsA treated animals received 50 mg/kg of CsA on days -2, -1, and 0 and 4 μg/kg of rMuTNFα on days 0, 2, and 4. At day 7 the animals were sacrificed, the tumors enucleated and weighed. Experiment 4[b]: Tumor CC531 was implanted s.c.. Control animals received 0.1 ml HBSS i.l. on days 0, 5, 10, and 15. Control/CsA treated animals received 15 mg/kg CsA i.m. on days -2, -1, and 0 and three times a week thereafter until the end of the experiment and 0.1 ml HBSS i.l. on days 0, 5, 10, and 15. TNF-treated animals received 128 μg/kg of rMuTNFα(B) i.l. on days 0, 5, 10, and 15. TNF/CsA treated animals received 15 mg/kg of CsA i.m. on days -2, -1, 0, and from day 0 three times a week until the end of the experiment. At days 0, 5, 10, 15, 20, and 25 tumor diameter was measured.

Experiment 5: The importance of the effect of TNFα on tumor vessels and clotting was assessed in a SRCA. Clotting was prevented by pretreating the animals with heparin. Tumor CC531 was implanted in a SRCA. Control animals received 1 ml HBSS i.v. on days 0, 2, and 4. TNF treated animals received 4 μg/kg of rMuTNFα(F) on days 0, 2, and 4. TNF/heparin treated animals received 75 U heparin/animals

prior to TNFα. At days 0, 2, and 4 they received 4 μg/kg of rMuTNFα(F). At day 7 the animals were sacrificed, the tumors enucleated and weighed. The effect of TNFα on the number of tumor vessels was counted by use of luconyl blue.

RESULTS

In vitro sensitivity for rMuTNFα (Figure 2.1):

Cells of the CC531 tumor were highly resistent to the cytostatic/cytolytic activity of rMuTNFα as determined by the MTT assay. Only at a dose of 5000 U/ml TNFα caused a significant reduction in the number of tumor cells after 48 hours of incubation ($p < 0.05$).

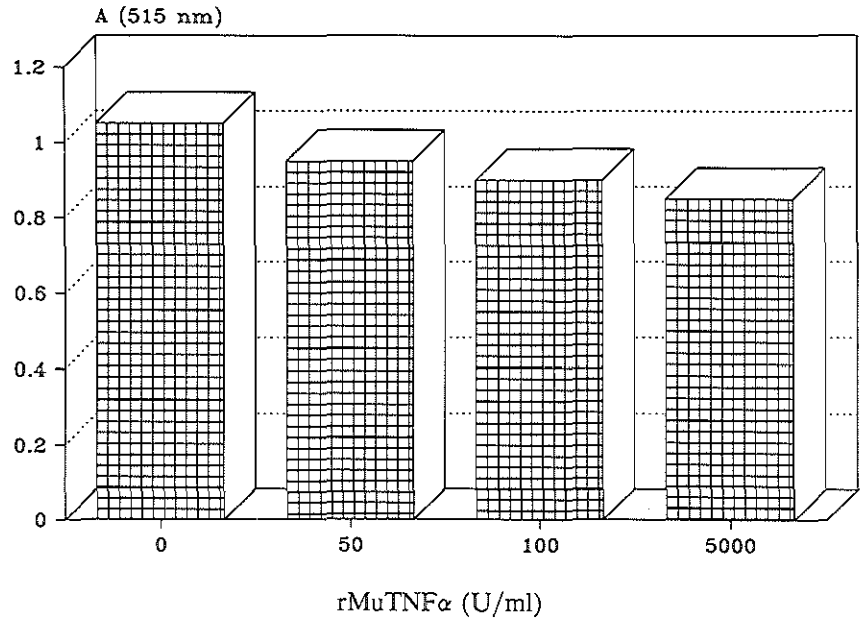

Figure 2.1 Effect of different amounts of rMuTNFα on in vitro growth of tumor cell line CC531, after incubation for 48 hours. Viability was measured using the MTT-assay, and absorbtion (A) was read at 515 nm. The difference between the control group and the groups treated with 5000 U/ml of rMuTNFα was statistically significant ($p<0.05$).

rMuTNFα toxicity in vivo:

When given as a single dose of 40 μg/kg of rMuTNFα(F) in 1 ml HBSS, all animals were in a near-death condition after about 5 to 6 hours and then killed (see also Marquet et al. 1987). The animals had diarrhea after about 3 hours, blood appearing in the faeces rapidly thereafter. At autopsy, the most prominent finding was hemorrhagic enteritis which was mainly restricted to the cecum and the colon. When given as continuous infusion, animals tolerated doses up to 200 μg/kg/day for 4 days well, but all animals receiving 400 μg/kg/day, died within 27 hours. Animals dying after receiving 400 μg/kg/day did not have diarrhea and the colon and cecum appeared to be normal at histopathologic examination.

Antitumor effect of rMuTNFα in a SRCA (Table 2.1):

When given systemically rMuTNFα(B), at a dose of 32 μg/kg, consistently inhibited the growth of tumor CC531. At a dose of 16 μg/kg rMuTNFα(B) did not inhibit growth of CC531.

Effect of TNFα on the number of liver metastases (Table 2.2 & 2.3, Figure 2.2 & 2.3):

Repeated injections of rMuTNFα(F) did not result in smaller numbers of liver metastases 28 days after tumor cell inoculation. Neither early treatment (administration of rMuTNFα(F) at days 0, 2, and 4 or at days 0 and 7) nor late treatment (administration of rMuTNFα(F) at days 14, 16, and 18 or at days 14 and 21) had any effect (Table 2.2, Figure 2.2).

When given as continuous infusion, rMuTNFα(F), at a dose of 4 μg/kg/day during days 0-4, did have a significant effect on the number of liver metastases ($p<0.01$). Giving the same dose during days 14-18 produced no significant reduction in the number of liver metastases. The group that received the sublethal dose of rMuTNFα(F) (200 μg/kg/day during days 14-18), also had no significant reduction in the number of liver metastases as compared to the control group (Table 2.3, Figure 2.3).

Table 2.1 Effect of rMuTNFα on growth of tumor CC531 in vivo

Treatment	Tumor growth (mg) mean ± SD	Tumor growth (mg) individual
Control	9 ± 6	3, 4, 4, 7, 8, 13, 16, 20
16 μg/kg rMuTNFα(B)	3 ± 4	-2, -2, 1, 2, 5, 6, 7, 10
Control	22 ± 3	17, 17, 21, 21, 23, 23, 24, 25, 25, 27
32 μg/kg rMuTNFα(B)	15 ± 6*	6, 7, 15, 16, 19, 19, 20, 21

* Significantly different from control ($p<0.01$ according to Wilcoxon's Rank Sum Test).
In vivo sensitivity of tumor CC531 for rMuTNFα or rHuTNFα treatment. A sub-renal-capsule assay was performed in rats. Control rats received i.v. 1 ml HBSS on days 0, 2, and 4. Day 0 is the day of tumor implantation. rMuTNFα-treated rats received either 16 or 32 μg rMuTNFα(B)/kg i.v. on days 0, 2, and 4. Significant growth inhibition was consistently observed when the animals were treated with 32 μg/kg of rMuTNFα ($p<0.01$). Each treatment protocol has been tested at least twice with each time the same result; the results of only one experiment are shown.

Tumor growth inhibition of s.c. grown tumor CC531 by rMuTNFα (Figure 2.4):
rMuTNFα(B) inhibited dose dependly the growth of s.c. implanted tumor CC531, when administered i.l.. From day 10 onward rMuTNFα(B) treated tumors were significantly smaller than control tumors, regardless the dose of rMuTNFα(B) used. At day 25 the tumors of the animals that were treated with 128 μg/kg of rMuTNFα(B) were significantly smaller than those of the animals treated with 64 μg/kg of rMuTNFα(B) ($p<0.05$ Wilcoxon's Rank Sum test).

Effect of TNFα on tumor growth in immunosuppressed animals (Table 2.4 & 2.5):
rMuTNFα had lost its activity partly on growth of tumor CC531 in a SRCA (Table 2.4) and completely on growth of s.c. implanted CC531. s.c. implanted CC531 grew significantly faster in immunosuppressed animals indicating that the tumor is indeed weakly immunogenic.

Table 2.2 Effect of rMuTNFα(F) on the number of liver metastases when given by i.v. injection.

Treatment	Mean score	n
Control[1]	2.50	10
Days 0, 2, 4[2]	2.90	10
Days 0, 7[3]	2.50	10
Control[4]	2.16	18
Days 14, 16, 18[5]	1.89	18
Days 14, 21[6]	1.39	18

[1]Control animals received 1 ml HBSS i.v. on days 0, 2, and 4 after tumor inoculation. [2]Animals received 4 μg/kg of rMuTNFα(F) i.v. on days 0, 2, and 4. [3]Animals recived 4 μg/kg of rMuTNFα(F) i.v. on days 0, and 7 after tumor inoculation. [4]Control animals received 1 ml HBSS i.v. on days 14, 16, and 18 after tumor inoculation. Animals recived 4 μg/kg of rMuTNFα(F) either on days 14, 16, and 18[5], or on days 14, and 21[6].

Effect of TNFα on tumor growth in a SRCA in animals pretreated with heparin:

In animals that were pretreated with heparin rMuTNFα(B) had still a significant growth inhibiting effect. No difference was observed between animals only treated with rMuTNFα(B) and animals pretreated with heparin and subsequently treated with rMuTNFα(B).

No difference was observed on histological preparations between the number of blood vessels per tumor area within the tumors of control animals and the tumors of rMuTNFα(B) treated animals.

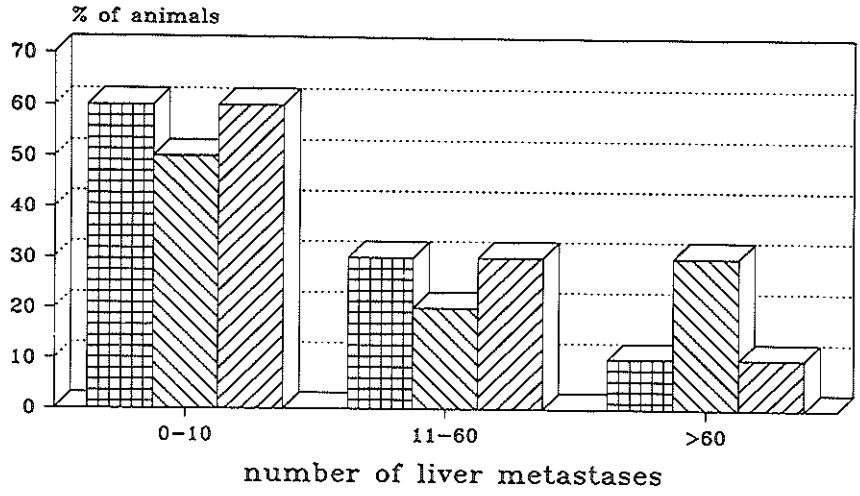

Figure 2.2[a]

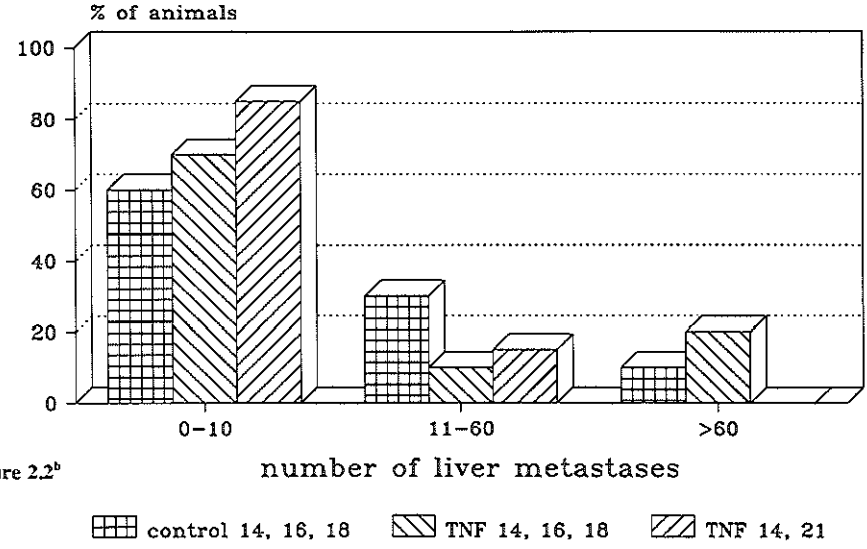

Figure 2.2[b]

Figure 2.2 Effect of repeated injections of 4 μg/kg of rMuTNFα on number of liver metastases. Results show the percentage of each group having 0-10, 11-60 and >60 metastases. Figure 2.2[a] shows the results of early treatment on day 0, 2, 4 and on day 0 and 7 vs control. Figure 2.2[b] shows the result of late treatment on day 14, 16, 18 and on day 14 and 21 vs control. Animals were inoculated on day 0, liver metastases were counted at day 28. The difference between control groups and rMuTNFα-treated groups was not statistically significant.

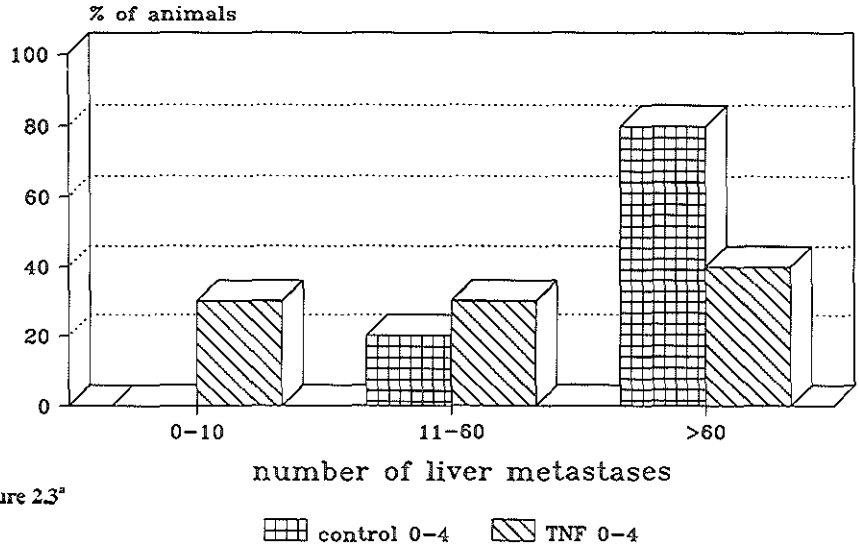

Figure 2.3[a]

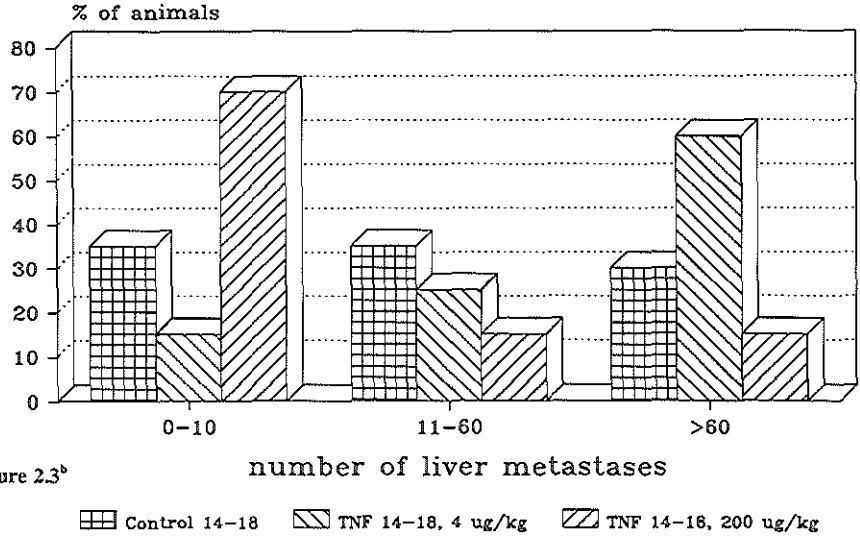

Figure 2.3[b]

Figure 2.3 Effect of continuous infusion of 4 μg/kg/day and 200 μg/kg/day of rMuTNFα on the number of liver metastases. Results show the percentage of each group having 0-10, 11-60, and >60 metastases. Figure 2.3[a] shows the results of early treatment during days 0-4 vs control. Figure 2.3[b] shows the results of late treatment during days 14-18. Animals were inoculated on day 0, liver metastases were counted at day 28. Only the difference between the control group and the group treated during days 0-4 was statistically significant ($p<0.05$).

Table 2.3 Effect of rMuTNFα(F) on the number of liver metastases when given by continuous infusion.

Treatment	Mean score	n
Control[1]	4.60	11
Days 0-4[2]	3.60[6]	9
Control[3]	3.50	14
Days 14-18, 1[4]	3.00	6
Days 14-18, 50[5]	2.67	6

[1]Control animals received10 ml HBSS i.v. by continuous infusion on days 0-4. [2]Animals received 4 μg/kg rMuTNFα(F) dissolved in 10 ml HBSS i.v. by continuous infusion on days 0-4. [3]Control animals received 10 ml HBSS i.v. by continuous infusion on days 14-18. Animals received either 4 μg/kg[4] or 200 μg/kg[5] of rMuTNFα(F) i.v. by continuous infusion on days 14-18. [6]Significantly different from control ($p<0.05$ according to the Chi-square test for means).

DISCUSSION

From the literature it is known that transformed cell lines respond differently to TNFα treatment. TNFα causes cytolysis or cytostasis in some cell lines, while other cell lines do not respond at all to TNFα treatment (Sugarman et al. 1985, Nio et al. 1988, Fransen et al. 1986). What determines whether a cell will respond to TNFα or not is not fully understood. Colon carcinoma CC531 appeared to be highly resistent to the cytostatic/cytolytic effects of TNFα in vitro as was demonstrated by the fact that even at a concentration of 5,000 U/ml, TNFα had only a marginal effect on tumor growth (85% survival).

TNFα treatment did inhibit growth of tumor CC531 in vivo. Systemic and intralesional administration of rMuTNFα resulted in growth inhibition in a SRCA and in subcutaneously grown CC531. Occasionally intralesional administration even led to tumor regression. Increasing the efficacy of i.v. administered TNFα by

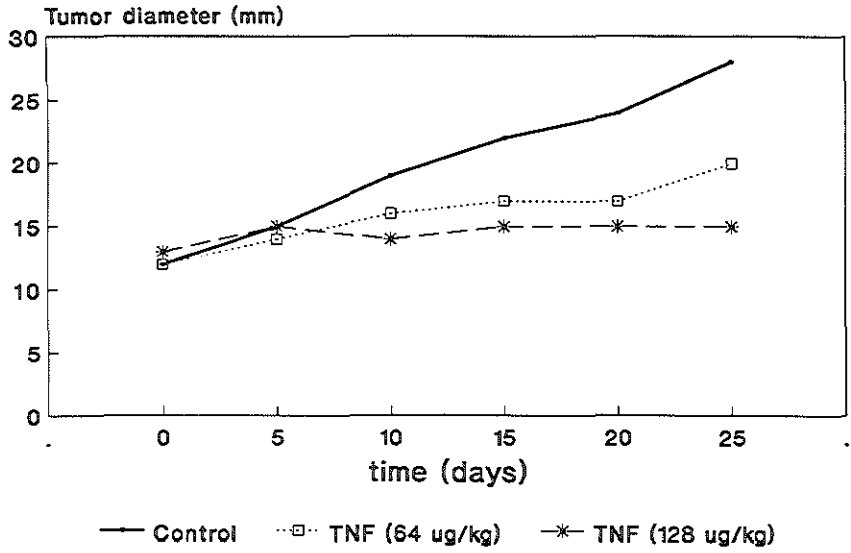

Figure 2.4 Effect of rMuTNFα(B) on growth of tumor CC5331 when grown subcutaneously. Control animals received 0.1 ml HBSS on days 0, 5, 10, and 15 i.l. TNFα treated animals received either 64 μg/kg or 128 μg/kg rMuTNFα(B) i.l. on days 0, 5, 10, and 15 dissolved in 0.1 ml HBSS. Tumor diameter (mm) was measured at days 0, 5, 10, 15, 20, and 25. From day 10 onward the difference between the control group and the rMuTNFα(B) treated animals was statistically significant (Wilcoxon's Rank Sum test). At day 25 the difference between the animals treated with 64 μg/kg and 128 μg/kg was significant as well ($p<0.05$ according to Wilcoxon's Rank Sum test).

increasing the dose could not be done because of unacceptable toxicity. This close relationship between the curative dose and the lethal dose has also been reported by others (Haranaka et al. 1984).

One possible way to increase the dose of TNFα without increasing toxicity might be administration via continuous infusion instead of administration via repeated bolus injections. Indeed this study shows that via continuous infusion 200 μg/kg, that is 5 times the lethal single injection dose, could be administered daily for 4 days, without incurring major problems. At a dose of 400 μg/kg/day all animals died within 27 hours probably because of shock. However, even at a sublethal dosage, i.e. 200 μg/kg/day, rMuTNFα(F) had no significant effect on growth of tumor CC531, when present as established tumor, in a liver metastases model. Therefore we concluded that administration of TNFα via continuous infusion is not preferable to repeated

Table 2.4 Effect of rMuTNFα(F) in immunosuppressed rats on tumor CC531 in a SRCA

Treatment	Tumor weight (mg) mean ± SD	Tumor weight (mg) individual
Control[1]	25 ± 7	11, 20, 21, 25, 26, 27, 30, 36
Control CsA[2]	22 ± 7	12, 17, 20, 21, 23, 25, 25, 36
TNFα[3]	10 ± 3[5]	7, 8, 8, 8, 10, 11, 12, 15
TNFα, CsA[4]	16 ± 4[6]	7, 13, 16, 16, 16, 17, 19, 20

[1]Control animals received 1 ml HBSS i.v. on days 0, 2, and 4 after tumor implantation. [2]Animals received 50 mg/kg CsA i.m. on days -2, -1, and 0 and 1 ml HBSS i.v. on days 0, 2, and 4. [3]Animals received 4 μg/kg of rMuTNFα(F) on days 0, 2, and 4. [4]Animals received 50 mg/kg of CsA i.m. on days -2, -1, and 0 and 4 μg/kg of rMuTNFα(F) ondays 0, 2, and 4 after tumor implantation. At day 7 the animals were sacrificed, the tumors enucleated and weighed. [5]Significantly different from control ($p<0.01$ Wilcoxon's Rank Sum test). [6]Significantly different from control and from TNF treated animals ($p<0.05$ Wilcoxon's Rank Sum test).

bolus injections in inhibiting the growth of established tumors.

The fact that tumor CC531 is highly resistent to TNFα treatment in vitro, but sensitive in vivo indicates that TNFα's action must be indirect. The discrepancy between in vitro sensitivity and in vivo sensitivity of a tumor has been shown before (Brouckaert et al. 1986). In general the antitumor action of TNFα in vivo is supposed to depend on at least three mechanisms: a direct cytotoxic effect against tumor cells, an effect of TNFα on tumor vessels leading to hemorrhagic necrosis of the tumor center and, finally, an immunologic effect presumably responsible for total regression of the tumor (Havell et al. 1988). In most murine models used, hemorrhagic necrosis of the tumor center occurs after administration of TNFα (Carswell et al. 1975, Haranaka et al. 1984). In our rat model hemorrhagic necrosis of the tumor center was never observed, suggesting that this mechanism is of minor importance in this rat model. Hemorrhagic necrosis is a consequence of the actions of TNFα on tumor

Table 2.5 Effect of rMuTNFα(B) in immunosuppressed rats on subcutaneously grown CC531. Values presented are mean tumor diameters (mm) ± SD.

Time (days)	Control[1]	Control, CsA[2]	TNFα[3]	TNFα, CsA[4]
0	13 ± 2	13 ± 2	13 ± 3	12 ± 1
5	16 ± 2	17 ± 4	15 ± 3	16 ± 1
10	19 ± 3	22 ± 7	14 ± 3[5]	20 ± 1
15	19 ± 3	24 ± 7	12 ± 6[5]	22 ± 1
20	26 ± 4	31 ± 7	16 ± 8[5]	31 ± 2
25	30 ± 4	35 ± 9	15 ± 10[5]	36 ± 3

Treatment was started (day 0) when the diameter of the s.c. tumors was about 13 mm.
[1]Control animals received 0.1 ml HBSS i.l. on days 0, 5, 10, and 15. [2]Animals received 15 mg/kg of CsA i.m. on days -2, -1, and 0 and three times a week thereafter until day 25 and 0.1 ml HBSS i.l. on days 0, 5, 10, and 15. [3]Animals received 128 μg/kg of rMuTNFα(B) in 0.1 ml HBSS i.l. on days 0, 5, 10, and 15. [4]Animals received 15 mg/kg CsA i.m. on days -2, -1, and 0 and three times per week thereafter until day 25 and 128 μg/kg of rMuTNFα(B) i.l. on days 0, 5, 10, and 15. Tumor diameter was measured at day 0, 5, 10, 15, 20, and 25. The tumors of the TNF treated animals were significantly smaller than the tumors of the control animals from day 10 onward ([5]$p<0.01$ according to Wilcoxon's Rank Sum test). TNF had no tumor growth inhibiting effect when administered i.l. in immunosuppressed animals.

vessels. Because of this, various researchers have stressed the importance of this effect of TNFα. It has been shown that TNFα has far better antitumor effects on vascularized than on non-vascularized tumors (Carswell et al. 1975), and the clotting seen after TNFα treatment within tumor vessels has been reported to be essential for its antitumor action (Shimomura et al. 1988). Others, however, have demonstrated that beside the importance of the hemorrhagic necrotizing effect of TNFα, the immunomodulatory actions of TNFα are essential to obtain complete cures. It appeared that TNFα only effected the growth of immunogenic tumors, while non-immunogenic variants were resistent to TNFα's actions (Asher et al. 1987).

Table 2.6 Effect of rMuTNFα(F) on growth of tumor CC531 in rats pretreated with heparin.

Treatment	Tumor growth (mg) mean ± SD	Tumor growth (mg) individual
Control[1]	22 ± 3	17, 17, 21, 21, 23, 23, 23, 24, 25, 25, 27
TNFα[2]	15 ± 6[4]	6, 7, 15, 16, 19, 19, 20, 21
TNFα, Heparin[3]	18 ± 8[4]	10, 10, 12, 13, 15, 16, 18, 23, 25, 35

[1]Control animals received 1 ml HBSS i.v. on days 0, 2, and 4 after tumor implantation. [2]Animals received 32 μg/kg of rMuTNFα(B) on days 0, 2, and 4. [3]Animals received 75 IE/animal immediately before rMuTNFα(B) administration; they also received 32 μg/kg of rMuTNFα(B) i.v. on days 0, 2, and 4. At day 7 the animals were sacrificed, the tumors enucleated and subsequently weighed. [4]Significantly different from control ($p<0.05$).

Furthermore it was demonstrated that TNFα had better antitumor effects in heterozygous (nu/+) littermates than in homozygous (nu/nu) nude mice, despite the fact that hemorrhagic necrosis of the tumor was observed in both mice (Haranaka et al. 1984). T-cells seemed to be important in this respect because in normal mice, pretreated with L3T4 or Thy-1 monoclonal antibodies, resulting in depletion of (helper) T-cells, the ability of TNFα to induce tumor regression was completely abrogated (Palladino et al. 1988). In our rat model the immunomodulatory effects of TNFα seem to be of greater importance than its effect on tumor vessels or on clotting. Immunosuppression of the rats resulted in partial (SRCA) or complete (s.c. model) abrogation of the tumor growth inhibiting effect of TNFα. T-cells are probably involved in this rat model because CsA is supposed to affect mainly the T-helper population. No effect was observed on the antitumor action of TNFα of heparin pretreatment, suggesting that clotting is of no importance in our rat model. This is also in agreement with the results of Bloksma et al. (1984), who observed that heparin pretreatment did not interfere with the antitumor action of endotoxin against MethA sarcoma's. Another clue that the action of TNFα on tumor vessels is less important in our model is that the number of tumor vessels per tumor area, as shown

with luconyl blue staining, was the same in control animals and experimental animals.

In conclusion: rMuTNFα can inhibit growth of colon carcinoma CC531 in vivo despite the fact that it is highly resistent to rMuTNFα treatment in vitro. TNFα exerts this effect by modulation of the immune system rather than via an effect on tumor vasculature. I.l. administration is superior to i.v. administration in terms of efficacy. Increasing the i.v. dosage by giving TNFα via continuous infusion does not result in better antitumor efficacy of TNFα on tumor CC531 in a liver metastases model. Treatment with TNFα never resulted in complete cures, indicating that TNFα is not too promising as antitumor agent when used in single agent therapy. Therefore we propose to search for other agents that might act additive or even synergistic with TNFα in this respect. These agents might be other cytokines, such as interferons.

CHAPTER 3

ANTITUMOR EFFECT OF INDUCED ENDOGENOUS TNFα ON TUMOR GROWTH; A STUDY IN RATS

This chapter is an adapted version of the article

The antitumour activity of the interferon inducer bropirimine
is partially mediated by endogenous tumour necrosis factor-α
by Marcel Scheringa, Jan N.M. IJzermans, Johannes Jeekel, and Richard L. Marquet

Cancer Immunol. Immunother. 32: 251-255, 1990

SUMMARY

Pyrimidinones, like 2-amino-5-bromo-6-phenyl-4-pyrimidinone (Bropirimine, ABPP), are potent immunomodulators. Natural Killer cell activity and macrophage cytotoxicity are increased after ABPP treatment. ABPP exerts this effect through induction of cytokines. Up to now, the interferons were supposed to be the main mediators but we found that TNFα can also be an important mediator of the ABPP antitumor effects. Increased serum levels of TNFα were seen in rats after intraperitoneal administration of 200 mg/kg of ABPP on two consecutive days. We also found that the tumor growth inhibiting effect of ABPP on a colon carcinoma in rats could be reduced about 40% by giving the rats rabbit-anti-TNFα serum just prior to ABPP treatment.
These results indicate that ABPP can induce the release of TNFα in vivo and that this endogenous TNFα may be important as far as the antitumor effect of ABPP is concerned.

INTRODUCTION

Immunotherapy is a promising modality in the treatment of cancer. Treatment with cytokines, sometimes in combination with adoptive transfer of immunocompetent cells, is successful in several animal models as well as in patients with different types of malignancies, especially renal cell carcinoma or melanoma (Borden et al. 1984, Talmadge et al. 1987, Winkelhake et al. 1987, Rosenberg et al. 1988, Pfreundschuh et al. 1989). The cytokines interleukin-2, the interferons, and tumor necrosis factor alpha (TNFα) have been most frequently studied. We have been working for some years with interferon gamma and TNFα in various experimental tumor models (IJzermans et al. 1987, Marquet et al. 1987, Marquet et al. 1988). TNFα appeared to have a growth inhibiting effect in some models (Marquet et al. 1987, 1988), but in those models resembling the clinical situation best, i.e. liver metastases of colon carcinoma, a significant growth inhibition could not be demonstrated despite usage of different administration schedules (see chapter 2). Therefore we, and others, concluded that

TNFα should be used in combination therapy, for example with other biological response modifiers, and it has indeed been demonstrated that combining TNFα with IFN-gamma or alpha results in synergistic antitumor effects (Balkwill et al. 1986, Yamaue et al. 1987, Agah et al. 1988, Shah et al. 1989).

Another suggested possibility of increasing the efficacy of exogenous TNFα, is to administer it at the time endogenous TNFα is released. This therapy has been referred to as endogenous-exogenous therapy (EET, Inagawa et al. 1988). Examples of TNFα-inducing agents are endotoxin (LPS) and OK432. The former is a strong TNFα inducer but can hardly be used clinically due to severe toxicity, while the latter is a strong and far less toxic TNFα inducer (Kato et al. 1985, Sekimoto et al. 1988).

In search for other TNFα inducing agents, the known interferon inducer Bropirimine (ABPP) was tested in vivo. We examined whether it can cause TNFα release by measuring serum levels of TNFα after intraperitoneal (i.p.) administration of ABPP in rats. Furthermore, we examined the effect of ABPP treatment on a colon carcinoma and a rhabdomyosarcoma as compared to TNFα treatment. Finally, we examined whether the ABPP antitumor effect could be blocked by pretreatment with rabbit-anti-TNFα serum.

MATERIALS AND METHODS

Animals:

Male rats of the inbred WAG ($RT1^u$) strain were used (for details see chapter 2).

Tumors:

Tumor CC531 and R2K were used (for details of tumor CC531 see chapter 2). R2K is a rhabdomyosarcoma transplantable in syngeneic WAG rats (Reinhold et al. 1966). R2K was maintained in vitro in Dulbecco's modified Eagle medium supplemented with 10% newborn calf serum (the serum was screened for virus and Mycoplasma infections). This medium was supplemented with 1% penicillin (5,000 U/ml), 1% streptomycin (5,000 U/ml) and 1% L-glutamine (200 mMol), all obtained from Gibco

(Paisley, UK). Before use, the cells were trypsinized (5 min, 37°C), centrifuged (5 min, 700g), resuspended in medium and counted. Viability was measured using Trypan blue (0.3% in a 0.9% NaCl solution); it consistently exceeded 95%.

In vitro testing of tumor cell lines for response to TNFα:

Tumor cells were seeded at 1 x 10^4 cells per well in flatbottomed 96-well microtiter plates (Costar, Cambridge, MA) in a final volume of 0.2 ml medium per well, and incubated at 37°C in 5% CO_2 for 48 hours in the presence of different concentrations of recombinant murine tumor necrosis factor α (rMuTNFα). Concentrations of rMuTNFα were 0 U/ml, 50 U/ml, 100 U/ml and 5000 U/ml. Growth of tumor cells was measured using an MTT-assay (for details see chapter 2). Absorbance was read at 515 nm using a Flow Titertek Multiscan (McLean, VA) plate reader. Eight replicate wells were used for each concentration tested.

Agents:

In these experiments rMuTNFα(F) was used (see chapter 2).

2-amino-5-bromo-6-phenyl-4-pyrimidinone (Bropirimine, ABPP) was produced as reported previously (Wierenga et al. 1980) and provided by the Upjohn Company, Kalamazoo, Michigan, USA.

Rabbit-anti-mouse-recombinant TNFα serum (RaTNFS) was prepared in our laboratory. Rabbits were three times immunized with rMuTNFα in combination with Freund's adjuvants (25 μg rMuTNFα/immunization, administered intracutaneously). Blood was collected one week after the last immunization, serum was stored and tested for anti-TNFα activity. RaTNFS had a neutralizing capacity of 100,000 U/ml serum in the L929 bioassay (see next paragraph). In vivo 0.5 ml of RaTNFS given intravenously (i.v.) protected rats against the lethal effect of 20 μg rMuTNFα(F) given i.v. subsequently. Non RaTNFS pretreated rats (n=2) both died while RaTNFS pretreated rats (n=2) survived after rMuTNFα(F) administration without signs of toxicity.

TNFα assay:

For measurement of TNFα in the serum samples of the rats a standard cytotoxicity assay for TNFα, using the L929 cell line, was performed. Cells were seeded at $5x10^5$ cells per well (100 μl) in flat bottomed 96-well microtiter plates in the presence of Actinomycin-D (final concentration 1 μg/ml). Sera, 50 and 100 times diluted, were added (to a final volume of 200 μl) and after 20 hours, cell survival was estimated by the colorimetric MTT assay. Absorbance was read at 515 nm. The TNFα concentrations were calculated by comparison with a standard curve.

Experimental design:

To test the sensitivity of CC531 and R2K for TNFα and ABPP in vivo, a sub-renal capsule assay (SRCA, see chapter 2) was performed. The animals were sacrificed at day 7, the tumors were enucleated and subsequently weighed.

Treatment consisted of i.v. injections of 4 μg/kg of rMuTNFα(F) (rMuTNFα treatment) on days 0, 2 and 4 or i.p. injections of 200 mg/kg of ABPP (ABPP treatment) on days 0 and 1. Control animals received Hanks Balanced Salt Solution (HBSS) i.v. and Phosphate Buffered Saline (PBS) i.p. (Control treatment). Each treatment was tested twice on both tumors.

In order to study the TNFα-inducing capacity of ABPP, rats received 200 mg/kg of ABPP i.p. on two consecutive days (the therapeutic regimen). During the second day blood samples were collected and serum stored at -20°C. Blood samples were taken 5 min prior to the second ABPP administration and after 1, 2, 3, 4 and 6 hours. Control animals received HBSS i.p.

In another series of experiments, using the SRCA model, we tested whether the antitumor effect of ABPP against an in vivo proven TNF-sensitive tumor (CC531) could be blocked by pretreatment with 0.25 ml of RaTNFS i.v. On days 0 and 1 the rats received 200 mg/kg of ABPP i.p. immediately after an i.v. injection of 0.25 HBSS, normal rabbit serum (NRS) or RaTNFS.

Statistical analysis:

For measurement of significance of difference in the SRCA, the Wilcoxon's Rank Sum test was performed.

For measurement of significance between control and ABPP treated groups in the TNFα-inducing experiments, the Student's t-test was used for the various time points.

RESULTS

In vitro sensitivity of CC531 and R2K for rMuTNFα:

Both cell lines were relatively insensitive to the cytostatic/cytolytic activity of rMuTNFα as measured after 48 h incubation (Figure 3.1). rMuTNFα caused only a minor, though significant, reduction in the number of viable cells in both cell lines at a concentration of 5000 U rMuTNFα/ml.

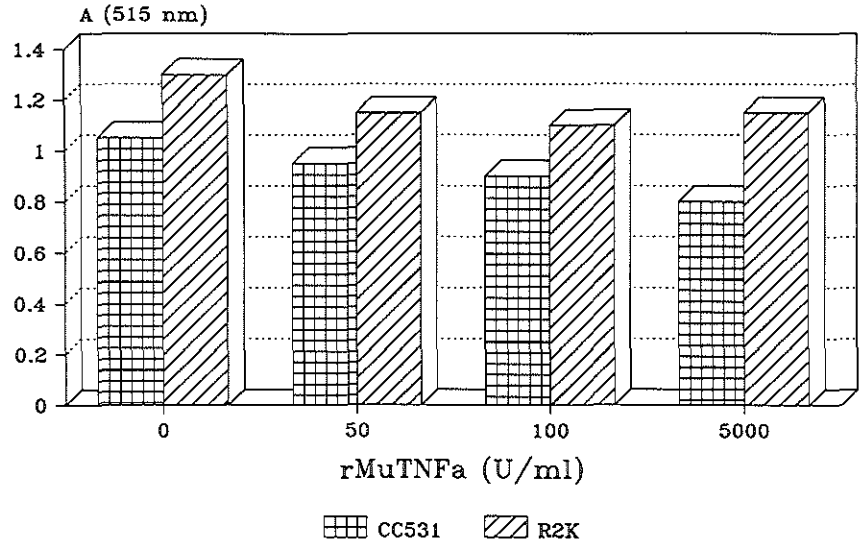

Figure 3.1 Effect of different concentrations of rMuTNFα (U/ml) on in vitro growth of tumor cell lines CC531 and R2K after incubation for 48 hours. Viability was measured using the MTT-assay; absorbance (A) was measured at 515 nm.

In vivo sensitivity of CC531 and R2K for rMuTNFα or ABPP:

RMuTNFα as well as ABPP did have a significant growth inhibiting effect on tumor CC531 while only ABPP could inhibit growth of tumor R2K. Agents were tested twice on each tumor (Table 3.1, results of one of the experiments is shown). Efficacy for both agents was in the same order of magnitude for colon cancer CC531.

Table 3.1 Sensitivity of CC531 and R2K for rMuTNFa or ABPP.

Tumor CC531 Treatment	Tumor weight mean ± SD (mg)	n	Tumor weight individual (mg)
Control	13 ± 3	12	8,9,10,11,12,13,14,16,16,16,17,19
rMuTNFα*	7 ± 3	12	2,4,6,7,7,7,7,8,9,9,11,12
Control	27 ± 11	10	10,18,22,22,24,24,26,32,35,52
ABPP*	10 ± 5	8	3,5,8,12,12,12,14,17
Tumor R2K Treatment	Tumor weight mean ± SD (mg)	n	Tumor weight individual (mg)
Control	26 ± 10	10	11,13,21,22,24,26,30,35,40,41
rMuTNFα	24 ± 9	10	11,14,17,18,26,28,29,32,33,37
Control	20 ± 5	12	11,14,15,17,17,19,21,22,24,25,26,28
ABPP*	12 ± 6	10	5,6,6,11,11,12,14,15,16,24

*significantly different from control.

In vivo sensitivity of tumors CC531 and R2K for rMuTNFα and ABPP. A SRCA was performed in rats. Control rats received iv 1 ml HBSS on days 0, 2 and 4 and 1 ml PBS ip on days 0 and 1 after tumor implantation. rMuTNFα treated rats received iv 4 μg rMuTNFα on days 0, 2 and 4. ABPP treated rats received i.p. 200 mg/kg of ABPP on days 0 and 1 after tumor implantation. Animals were sacrificed at day 7, tumors enucleated and weighed. Both rMuTNFα and ABPP had a significant growth inhibiting effect on tumor CC531 ($p<0.01$ and $p<0.05$ respectively). Growth of tumor R2K was significantly inhibited by ABPP ($p<0.01$) but rMuTNFα did not inhibit growth of R2K.

In vivo TNFα induction by ABPP:

Administration of 200 mg ABPP/kg i.p. on two consecutive days (the therapeutic regimen) resulted in a significant increase in TNFα serum levels (Figure 3.2). TNFα serum levels peaked at 1 hour after the second administration and remained significantly elevated for up to 6 hours at least. Mean TNFα serum levels in controls were surprisingly high at t=5 min because two of the control rats had unexplained high TNFα starting levels.

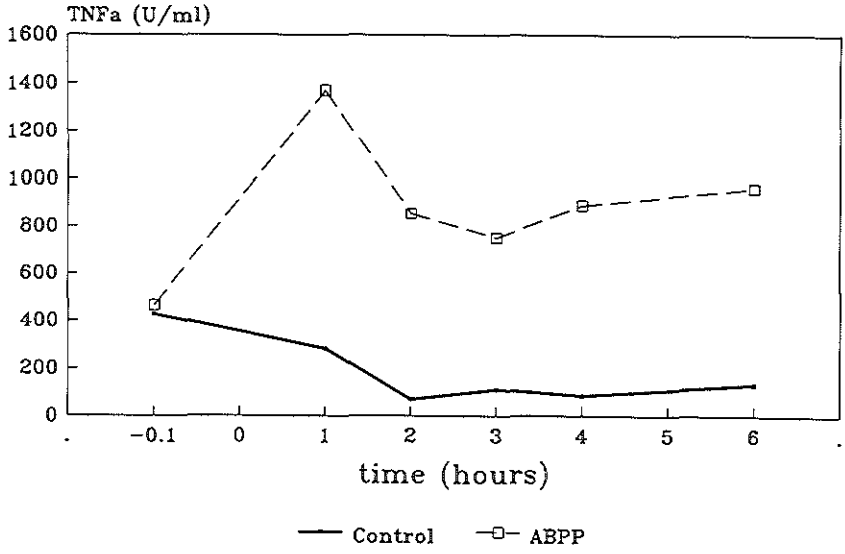

Figure 3.2 Serum TNFα levels in rats after treatment with ABPP (200 mg/kg i.p. on two consecutive days) or after treatment with HBSS (1 ml i.p. on two consecutive days). Samples were taken 5 minutes prior to the second administration and 1, 2, 3, 4 and 6 hours thereafter. TNFα was measured using the L929 bioassay. At 1, 2, 3, 4 and 6 hours TNFα serum levels were significantly increased ($p<0.005$ for each time point) in the ABPP treated group as compared to HBSS controls.

Blocking of the antitumor effect of ABPP against CC531 in vivo by RaTNFS:

In two experiments RaTNFS partially blocked the tumor growth inhibiting effect of ABPP on CC531 (Table 3.2). In the first experiment, the effect of ABPP + RaTNFS was 59% of the effect of ABPP + HBSS, while in the second experiment the effect of ABPP + RaTNFS was 57% of the effect of ABPP + NRS.

Table 3.2 Blocking of the antitumor effect of ABPP on coloncancer CC531 by pretreatment with RaTNFS.

Pretreatment/ treatment	Tumor growth mean ± SD (mg)	n	Tumor growth individual (mg)
Control/control	25 ± 1	12	2,13,18,25,25,25,26,26,27,31,31,51
Control/ABPP*	4 ± 2	10	0,1,3,4,5,5,5,6,6,6
RaTNFS/ABPP**	13 ± 8	12	0,0,1,9,13,13,14,18,19,21,22,22
Control/control	13 ± 5	12	5,7,7,7,10,14,14,14,17,17,20,20
NRS/ABPP*	3 ± 4	6	-2,-1,1,5,5,7
RaTNFS/ABPP***	7 ± 5	8	1,2,2,4,11,11,12,12

*significantly different from control/control

**significantly different from control/control and from control/ABPP

***significantly different from control/control and from NRS/ABPP

In two separate experiments the effect of pretreatment with RaTNFS on antitumor effect of ABPP on tumor CC531 in a SRCA was measured. Animals were pretreated with HBSS (control), normal rabbit serum (NRS) or rabbit anti TNFα serum (0.5 ml i.v. just prior to treatment), and then treated with HBSS (control, 1 ml i.p.) or ABPP (200 mg/kg i.p.) at days 0 and 1 after tumor implantation. After 7 days animals were sacrificed, tumors enucleated and weighed. Tumor growth was calculated. In both experiments groups treated with ABPP, regardless of pretreatment were significantly different from the HBSS treated groups ($p<0.01$). In both experiments the groups pretreated with HBSS or NRS and subsequently treated with ABPP were significantly different from the groups pretreated with RaTNFS and treated with ABPP ($p<0.01$).

DISCUSSION

Although it has been described that ABPP induces the release of lymphokines other than IFN as well (Richard et al. 1987), its TNFα inducing capacity has never been emphasized before. This capacity could be very interesting in the light of its role in

tumor therapy. So far, the antitumor effects of ABPP were believed to be especially mediated by endogenous interferons, leading to increased NK-cell and macrophage cytotoxicity (Lotzova et al. 1984, Eggermont et al. 1986). The results of this study suggest that endogenous TNFα can also be an important effector of ABPP activity.

In rats, 200 mg/kg of ABPP given i.p. on two consecutive days resulted in increased serum levels of TNFα. All rats in the ABPP treated group had high starting levels of serum TNFα, indicating that single ABPP administration does also induce TNF release (TNFα serum levels were only measured after the second ABPP administration). The high mean TNFα serum levels in the control group at t=5 min, were due to two of the control animals. These animals had persistently higher TNFα levels throughout the experiment than the other rats in the control group in whose serum virtually no TNFα could be demonstrated. The tumor growth inhibiting effect of ABPP against an in vivo proven TNFα sensitive tumor, could be reduced about 40% by pretreatment with rabbit-anti-TNFα serum. This study shows that TNFα is only one of the mediators through which ABPP exerts its antitumor effect, since the in vivo TNFα-insensitive tumor R2K responded as well to ABPP as tumor CC531. ABPP has already been shown to be a potent immunotherapeutic agent against different types of experimental cancer (Milas et al. 1982, Eggermont et al. 1986), which is confirmed by this study. Treatment with ABPP often resulted not only in growth inhibition but even in tumor regression. Earlier studies in our laboratory have shown that bropirimine does not only inhibit growth of tumor CC531 in a SRCA but also in an artificial liver metastases model (Eggermont et al. 1986).

The TNFα inducing capacity of ABPP is the more interesting with regard to the so-called endogenous-exogenous therapy (EET). EET seems to have better antitumor efficacy than either induction of endogenous TNFα or administration of exogenous TNFα (Inagawa et al. 1988). So far, EET has been performed using the streptococcal preparation OK432 as the TNFα inducing agent (Sekimoto et al. 1988). Like ABPP, OK432 causes the release of TNFα (Saito et al. 1984), it increases macrophage (Saito et al. 1984), NK-cell (Yamaue et al. 1987), and large granular lymphocyte cytotoxicity (Uchida et al. 1984). It also induces the release of IFN (Saito et al. 1982). Peak levels of TNFα in serum of mice after OK432 treatment (Satoh et al. 1987) were in the

same order as peak levels of TNFα in serum of rats after ABPP treatment in this study. Although comparison of results obtained in rats and mice can never lead to final conclusions, ABPP might well be an alternative to OK432 in EET therapy. It has already been reported that ABPP and TNFα have synergistic antitumor effects (Marquet et al. 1984).

The recognition of ABPP as being a TNFα inducer in vivo, may well explain a previously reported effect of ABPP that could not be explained on the basis of its interferon inducing capacity. It appeared that ABPP could protect mice against listeriosis (Anthony et al. 1984). This protecting effect of ABPP against an infection with Listeria monocytogenes, remained when the mice were pretreated with a potent anti-interferon antibody, indicating that this effect was not due to endogenous interferon. It is known that endogenous TNFα protects mice against infection with Listeria, especially in the early stages of the infection (Havell 1987, Kratz et al. 1988), so the protecting effect of ABPP could well be mediated by endogenous TNFα.

From the results presented in this study it can be concluded that ABPP can induce the release of TNFα in rats; this endogenous TNFα can be important as far as the antitumor effects of ABPP are concerned and could make ABPP an even more important, immunomodulatory, antitumor agent.

CHAPTER 4

LOCAL TREATMENT OF LIVER METASTASES WITH RECOMBINANT HUMAN TNFα (rHuTNFα); A PHASE I STUDY.

This chapter is a modified version of the article

Immunotherapy with tumor necrosis factor:
Feasibility of ultrasound guided injection of rHuTNFα
into liver metastases
by J.N.M. IJzermans, G.P. van der Schelling, M. Scheringa, T.A.W. Splinter,
R.L. Marquet and J. Jeekel

Neth. J. Surgery 1991

SUMMARY

Fifteen patients with therapy-resistant liver metastases were treated in a phase I study with rHuTNFα. rHuTNFα was injected into one liver metastasis by ultrasound guidance, using a 50 μg escalating dose schedule (3 patients/dosage) ranging from 100 - 350 μg/injection. Influenza-like symptoms like fever, chills, nausea and vomiting were the main clinical side-effects. Other toxicities, as reported after systemic use of rHuTNFα, such as hypotension, decrease in leucocytes and platelet counts, renal or liver toxicity were not observed. In eight patients growth arrest was observed in rHuTNFα-treated metastases, whereas non-injected lesions showed growth progression. The MTD by this route of administration is > 350 μg/injection. Based on these observation we conclude that the toxicity of rHuTNFα injected into liver metastases by sonographic control is transient and mild and that intratumoral administration of rHuTNFα might play a role in local tumor control.

INTRODUCTION

TNFα was first identified in BCG-infected mice challenged with endotoxin. This serum factor caused hemorrhagic necrosis of subcutanously implanted meth A sarcoma in mice (Carswell et al. 1975). Subsequently it was demonstrated that TNFα is produced by activated cells of the monocyte/macrophage lineage (Männel et al. 1980). After the isolation of TNFα it was possible to identify the gene coding for this polypeptide (Pennica et al. 1984, Gray et al. 1984), and with the use of recombinant DNA technology TNFα came available in large amounts of highly purified material. It was thought that by the production of TNFα on a large scale the beginning was set for a selective form of immunotherapy in patients with advanced cancer.

The exact antitumor activity of TNFα is yet not clearly understood. Its direct cytotoxicity to sensitive tumor cells is mediated by specific cell surface receptors. Interaction of TNFα with these receptors leads to membrane perturbations and DNA fragmentation (Rubin et al. 1988). Malignant cell lines which exert no growth inhibition to TNFα in vitro, can show tumor regression in vivo. This implies that other, indirect, mechanisms may be involved in the antitumor activity of TNFα in vivo, such as activation of host

immune defense mechanisms (Balkwill 1989). Recently it was demonstrated that TNFα affects the hemostatic properties of vascular endothelium by stimulating procoagulant activity, thereby facilitating the formation of thrombi. TNFα can also damage vascular endothelial cells directly. Thus, within a solid tumor TNFα might lead to occlusion of its vessels and subsequent diminished perfusion which finally leads to necrosis of the tumor. Not only the neovasculature of certain tumors is sensitive to the activity of TNFα, but also normal tissue endothelium may be damaged (Bevilacqua et al. 1986, Sato et al. 1986).

Multiple phase I studies with TNFα have been carried out. In most studies TNFα was administered intravenously. Overall, it appears that systemic TNFα, in the doses and schedules examined, has little single agent activity in the treatment of advanced human malignancy. Due to excessive toxicity there is no further dose escalation possible. Dose-limiting toxicity mainly consisted of hypotension caused by the effects on normal endothelium by TNFα. Intratumoral application of TNFα, however, has occasionally resulted in local tumor regression. The lesions were mainly located superficially and therefore easy to inject. By this route higher concentrations can be achieved at the site of malignancy, without severe systemic toxicity.

In the present study we report results of a clinical phase I trial of rHuTNFα administered into liver metastases by sonographic control of various types of adenocarcinomas.

MATERIAL AND METHODS

Patients:

Patients with histologically proven liver metastases refractory to standard anticancer therapy were eligible for protocol entry. Patients were informed about the design of the trial as a single arm, non-randomized, open label study to evaluate the toxicity of intratumoral rHuTNFα. Written informed consent was obtained from all patients treated. The study protocol had been approved by the institutional ethics committee.

Eligibility criteria included: age between 18 and 75 years, a Karnofsky performance status > 70, a life expectancy of more than 3 months, and an interval of more than 4 weeks

since any prior antitumor therapy; a normal renal (creatinine < 110 umol/l) and hepatic function (bilirubin < 20 umol/l and SGOT < 90 U/l); a normal peripheral blood count, including hematocrit > 25%, leucocytes > 3 x 10^9/l, and platelets > 100 x 10^9/l.
Excluded were patients with uncontrolled infection, cardiac disease, hypertension, pregnancy, bleeding disorders or neurological disorders.
At entry of the study patients were evaluated by medical history, physical examination, Karnofsky performance status, coagulation profile, complete blood cell count, determination of liver and renal function, determination of serum electrolytes and urine-analysis.

Drug formulation:
rHuTNFα was provided in lyophilized form by Knoll AG, FRG. The product had a specific activity of 6.63 x 10^6 U/mg and was reconstituted in 2 ml of sterile 0.9% NaCl prior to injection. The preparation used was more than 99% pure and contained less than 10 pg of endotoxin per mg protein.

Ultrasound guided injection:
Before rHuTNFα administration a well-defined liver metastasis was selected with a sonography system (Aloka SSD 650, Japan). After the site of drug administration was anaesthetized with Xylocain 1%, rHuTNFα was injected into the selected liver metastasis with Shiba-needles (23 G) and sonographic control.

Treatment protocol and follow-up:
rHuTNFα was administered according to an 50 μg escalating dose schedule with three patients at each dose level. The study was started at a dose of 100 μg rHuTNFα/injection. Patients whose metastasis responded or remained stable, as measured after four weeks by sonographic control, received the same or a higher dose rHuTNFα until either progression of the metastasis or unacceptable toxicity occurred. If indicated, patients could be treated with non-steroid anti-inflammatory drugs (NSAID). Before rHuTNFα application and 1-4 days thereafter, the following parameters were monitored: hemoglobin, WBC and differential counts, platelets, SGOT, SGPT, LDH.

gamma-GT, total bilirubin, alkaline phosphate, creatinine, total serum protein, uric acid, prothrombin time, and urine-analysis. Vital signs (pulse, blood pressure and temperature) were controlled at 10, 20, 30 min, 1, 2, 4, and 8 hours and daily until the fifth day after rHuTNFα injection.
Grading of toxicity or response was determined as recommended by the World Health Organization (WHO, Miller et al. 1981).

Pharmokinetics and immunology:
Serum concentrations of TNFα were measured by IRMA (Medgenix, Fleurus, Belgium), with a detection level of 5 pg TNFα/ml serum. Peripheral blood samples were taken prior to rHuTNFα injection, and after 2, 5, 10, 20, 30, 60, and 120 minutes.
Subpopulations of lymphocytes were assessed by FACScan analysis using monoclonal-antibodies against several lymphocyte surface antigens (CD3, CD4, CD8, CD16, CD19).

Statistics:
Data before and after TNFα administration were compared using the paired Student's t-test. A p-value less than 0.05 was considered to indicate a significant difference.

RESULTS

Fifteen patients were entered into this study. Patient characteristics are listed in table 4.1. A total of 23 injections of rHuTNFα were administered and evaluable for toxicity. The same metastasis was injected two times in four patients and three times in two patients (Table 4.2). The main clinical side-effects included fever, chills, nausea, vomiting, diarrhoea, headache, and pain at the injection site (Table 4.3).

Table 4.1 Patient characteristics

total number of patients	15
sex m/f	11/4
age range (median) [years]	37-73 (60)
number of injections	23
previous therapy	
surgery	4
surgery + CT	4
surgery + RT	1
CT	4
RT	1
none	1
type of malignancy	
colorectal	9
pancreatic	2
gastric	2
hepatic	1
unknown	1
diameter of metastases (median) [cm]	2 - 16 (5.3)

CT = chemotherapy, RT = radiotherapy.

Systemic toxicity:

All patients treated by intratumoral injection of rHuTNFα experienced some degree of constitutional symptoms. Independent of the dose rHuTNFα chills appeared within 10-20 minutes after injection and lasted for 10-40 minutes. Although antipyretic drugs (indomethacin, paracetamol) were administered prior to 21 rHuTNFα injections in 13

patients, chills followed by fever developed after 13 injections in 9 patients, mild rigors without developing fever occurred after 4 injections in two patients, and fever without chills occurred in 2 patients. Two patients were not treated with NSAID and both developed chills followed by fever. Temperatures up to 39.9 degrees Celsius were found within 0.5-2 hours after rHuTNFα injection. Notably, some patients treated at higher doses did not experience chills or a rise in body temperature.
Hypotension requiring volume substitution was observed in two patients treated at doses of 200 μg and 300 μg, respectively. These patients had been treated concomitantly with morphine. In later courses the addition of morphine was avoided and similar or higher doses of rHuTNFα were not found to reduce blood pressure of the same patients.

Table 4.2 Dose of rHuTNFα in the six patients who received multiple injections.

patient	dose μg/inj					
	100	150	200	250	300	350
1	x	x				
2		x		x		
3				x	x	
4					xx	
5			x	x		x
6					xx	x

Hepatic toxicity:
There were no significant differences in liver function tests prior to and after rHuTNFα injection (figure 4.1). Changes in bilirubin, alkaline phosphatase, SGOT, SGPT, gamma-GT, and LDH, were not related to the size of the liver metastases treated or the dose of rHuTNFα injected.

Table 4.3 Side-effects of rHuTNFα injected into liver metastases.

dose μg/inj	100	150	200	250	300	350	total	%
x10^6 U/inj	0.66	0.99	1.32	1.65	1.98	2.31		
injections	3	3	3	3	6	5	23	
chills	2	3	3	3	5	3	19	84
fever[a]	3	2	3	2	5	3	18	78
nausea	2	2	1	1	4	2	12	52
vomiting	1	2	1	1	3	1	9	39
pain[b]	1	0	2	0	1	2	6	26
headache	1	1	2	0	0	0	4	18
diarrhea	0	0	0	0	1	2	3	13
hypotension[c]	0	0	1	0	1	0	2	9

[a] fever > 38° Celsius.
[b] pain at injection site; one patient (300 μg) had pain at the site of local recurrence in the sigmoid.
[c] hypotension < 100 mm Hg systolic.

Hematologic toxicity:

No effect of TNFα treatment was observed on RBC, WBC, WBC-differentials, and platelet count.

Pharmokinetics and immunology:

There were great variations in the maximum concentration and the time course of plasma levels TNFα in patients receiving the same dosage (Figure 4.2).

Lymphocyte-count and subpopulations of lymphocytes ($CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$, and $CD19^+$) in peripheral blood samples prior to rHuTNFα injection, and after 2 and 4 days showed no significant difference (Figure 4.3).

Figure 4.1 Liver function tests after various doses of rHuTNFα (100 - 350 μg) injected into liver metastases. Bilirubin = umol/l; alkaline phosphatase, SGOT, SGPT, gamma-GT, and LDH = U/l.

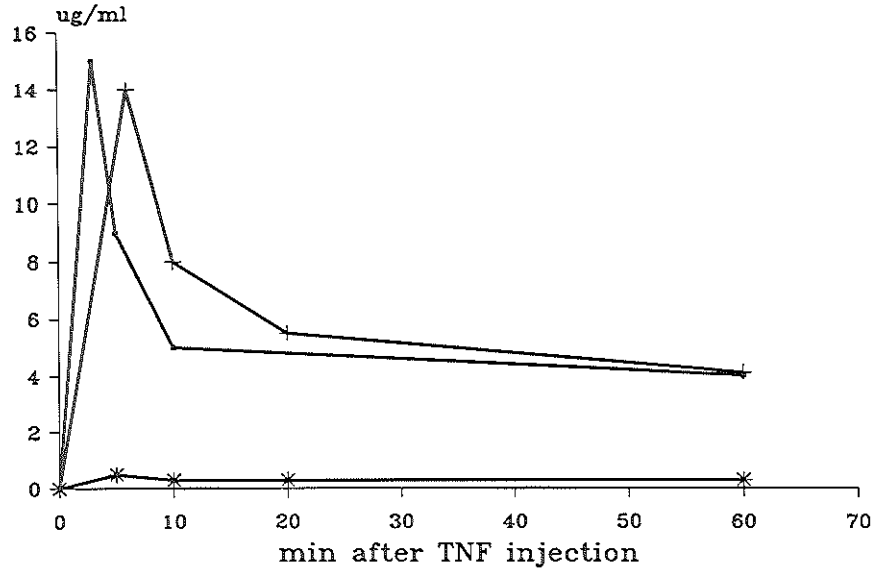

Figure 4.2 Serum concentration TNFα after intratumoral injection of 300 μg rHuTNFα.

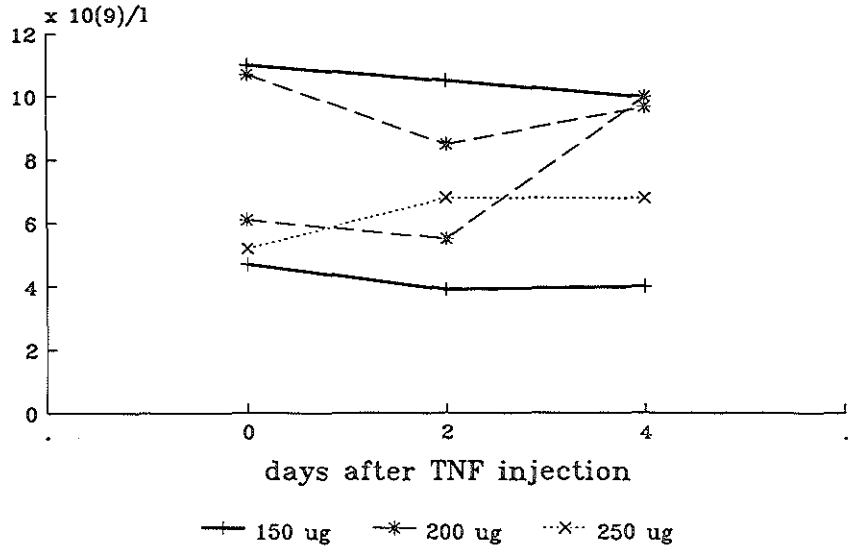

Figure 4.3[A] leucocyte count

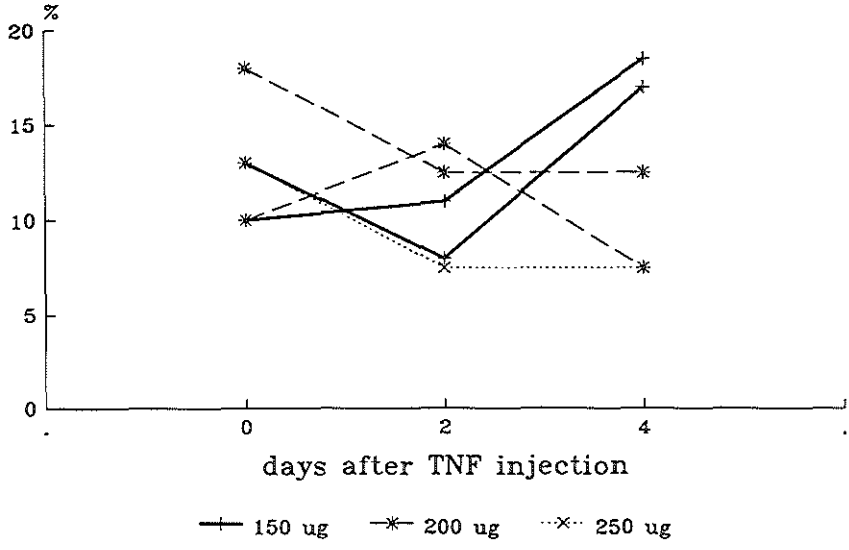

Figure 4.3[B] percentage of lymphocytes

Figure 4.3 Leucocyte count, percentage lymphocytes and lymphocyte subpopulations in peripheral blood samples prior to and after 2 and 4 days of TNFα injection.

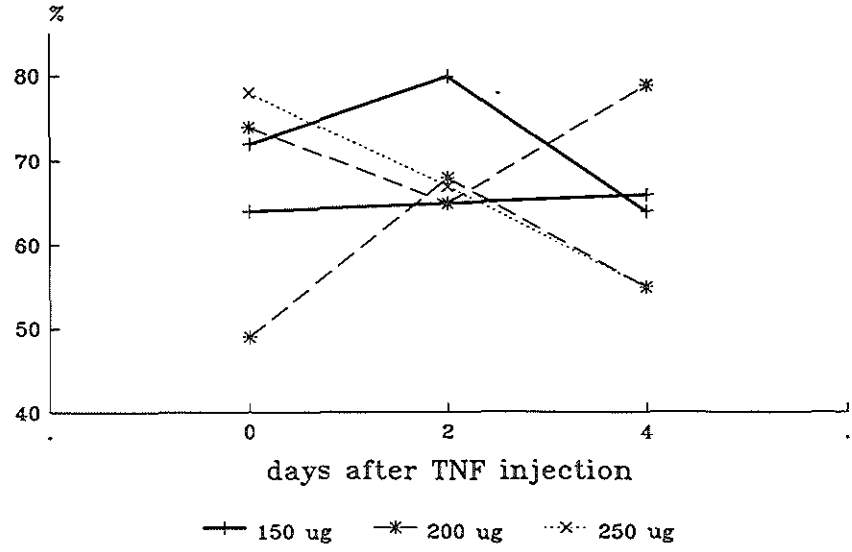

Figure 4.3[c] percentage of $CD3^+$ cells (T-cells)

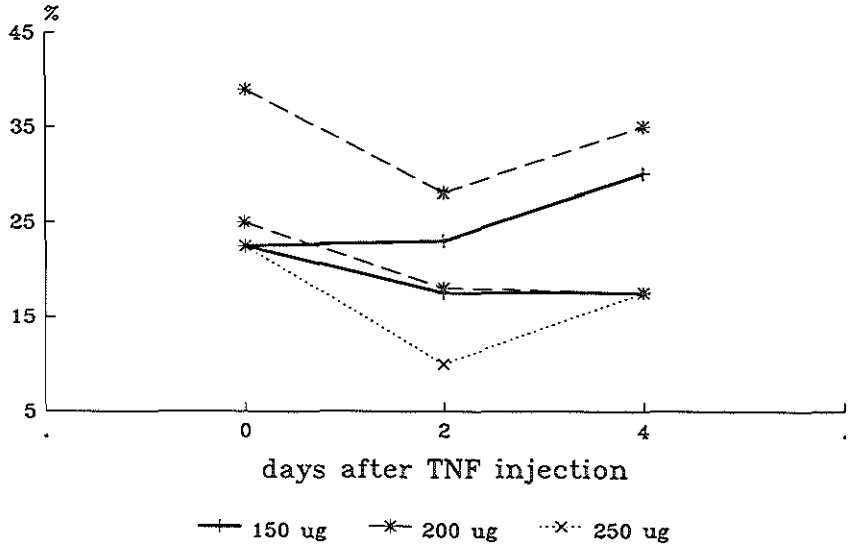

Figure 4.3[D] percentage of $CD8^+$ cells (T sup/cyt cells)

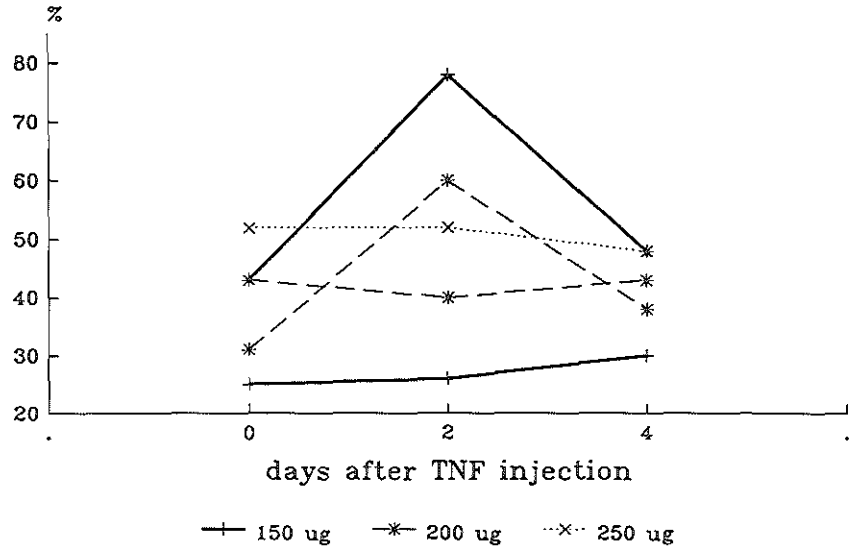

Figure 4.3[E] percentage of $CD4^+$ cells (Th cells)

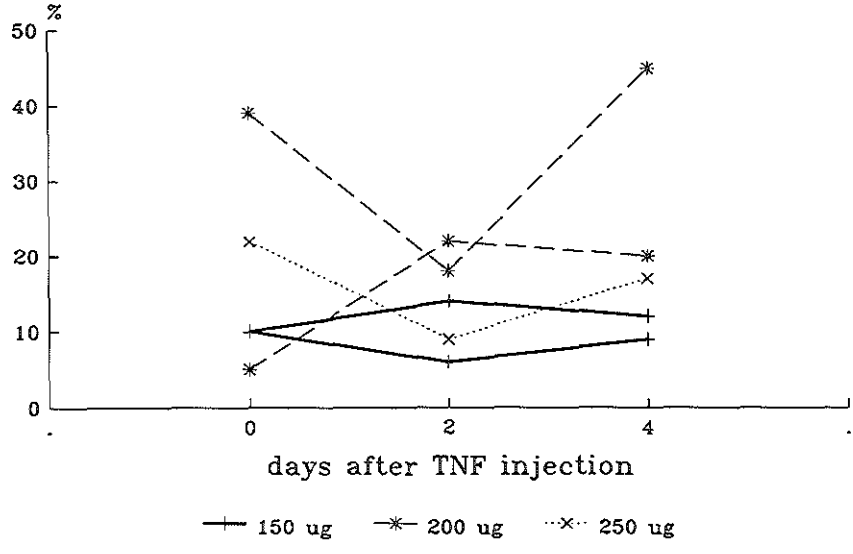

Figure 4.3[F] percentage of $CD16^+$ cells (NK-cells)

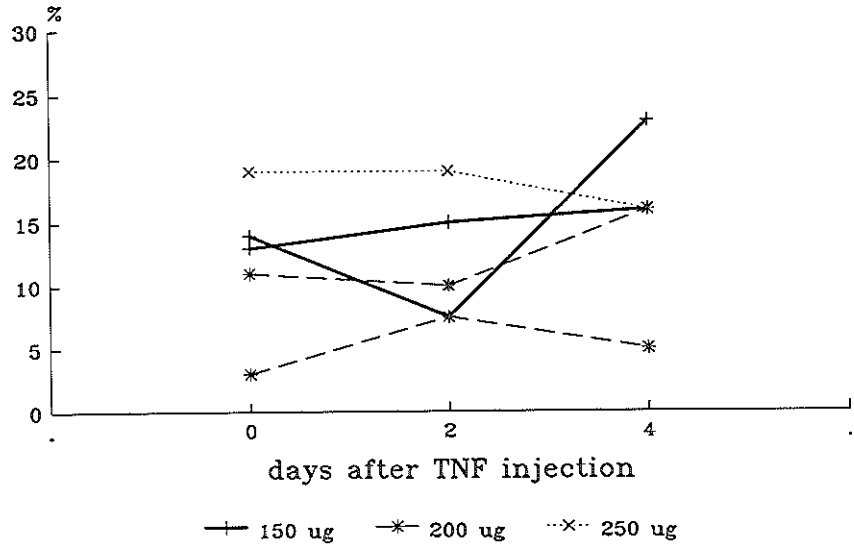

Figure 4.3[G] percentage of $CD19^+$ (B-Cells)

Other toxicity:

Five patients experienced pain at the site of injection, despite the use of local anaesthetics. After rHuTNFα administration into a liver metastasis one patient indicated pain at the site of a recurrent adenocarcinoma of the sigmoid. However, sonographic control of the local recurrence revealed no effect on tumor size. Gastro-intestinal effects as nausea, vomiting and diarrhea were mild and transient. Renal function as measured by serum creatinine remained unchanged.

Dose-limiting side-effects were not observed in any of the patients. As defined by the WHO criteria the most serious kind of complications observed in this trial can be indicated by grade 2 toxicity. All clinical side-effects were reversible within 12 hours after drug administration. The severity and duration of symptoms were not related to the dose of rHuTNFα injected.

Tumor response:

Stable disease was observed in eight patients. Characteristics of these patients are listed in table 4.4. It should be noted that in all patients only the liver metastasis treated with TNFα showed stable disease, while other metastases, located nearby within the same liver lobe, showed progression.

Table 4.4 Characteristics of responders.

patient	dose TNFα			diameter metastasis	response *	duration of response	type of malignancy
	1st inj	2nd inj	3rd inj				
62y ♂	100	-	-	6.3	Sd	4 weeks	pancreatic
44y ♂	100	150	-	9.7	Sd	4 weeks	unknown
52y ♂	200	-	-	6.3	Sd	4 weeks	colon
53y ♀	250	300	-	2.5	Sd	4 weeks	colon
53y ♂	300	-	-	4.0	Sd	4 weeks	colon
59y ♂	300	300	-	4.1	Sd	8 weeks	gastric
71y ♂	200	250	350	7.0	Sd	8 weeks	colon
63y ♂	300	300	350	7.6	Sd	8 weeks	colon

* = only response of injected metastasis, Sd = stable disease

DISCUSSION

The aim of this study was to establish the toxicity and safety of rHuTNFα injected into liver metastases by sonographic control. The main clinical side-effects were chills and fever despite pretreatment with indomethacin. No conclusions can be drawn of the effectiveness of indomethacin to attenuate toxicity. Although administration of indomethacin did not prevent side-effects, it may have reduced the severity and duration

of rHuTNFα toxicity. This assumption is invigorated by the results of a similar study where no NSAID were used to reduce toxicity. At a lower maximal dose, in comparison to our study, toxicity was more severe and resulted in life-threatening prostration (Pfreundschuh et al. 1989). Bartsch et al. (1989) reported that the severity and duration of chills, fever, local edema and pain at the injection site was dose related. Our study did not confirm this. Five patients experienced pain at the site of injection, although local anaesthetics were used prior to injection. One patient indicated pain at the site of a recurrent adenocarcinoma of the sigmoid. This phenomenon has also been reported by other investigators (Pfreundschuh et al. 1989). From results of the present study it is not clear whether these symptoms were caused by rHuTNFα itself, or maybe caused by other cytokines induced by TNFα, e.g. IL-1 (Dinarello et al. 1986). In our study hypotension was observed in two patients treated concomitantly with rHuTNFα and morphine. As both agents can induce a decrease of blood pressure and may act synergistically, concomitant use should be avoided. Hepatic toxicity is documented in several phase I studies of systemic administration of rHuTNFα (Sherman et al. 1988, Creaven et al. 1989, Jakubowski et al. 1989). Intratumoral application of a lower dose rHuTNFα, in comparison to this trial, showed elevations of SGOT or SGPT (> 2x baseline value) in 3 out of 21 patients, and elevation of bilirubin, alkaline phosphatase and gammaGT in 6 patients (Pfreundschuh et al. 1989). However, other investigators reported no deranged liver function after intratumoral application of TNFα, even at a higher dose than used in this trial (Bartsch et al. 1989). We also did not see any significant changes in liver function after TNFα injection in a liver metastasis. These controversial results may be related to the accuracy of the intratumoral rHuTNFα administration, and to the size and vascularization of the tumor, leading to differences in the rate of absorption and metabolization of the injected drug. This also applies for the differences in plasma levels TNFα measured after intratumoral injection of the same dosage. In other trials rHuTNFα was administered intravenously for 24 hours and lower plasma levels for a prolonged time were found (Spriggs et al. 1988, Steinmetz et al. 1988). It seems that the effect of rHuTNFα on the tumor depends mostly on its concentration at the tumor site, whereas side-effects are related to its duration in the plasma and not to the maximal plasma concentration. It is suggested that prolonged intravascular exposure to rHuTNFα

increases the number of TNFα-receptors on endothelial cells and that this mechanism explains the more severe side-effects seen by continuous intravenous administration of TNFα. It should be noted that others have found more serious side-effects, in comparison to our study, after an intravenous injection of a lower dose of rHuTNFα (Creaven et al. 1989, Kimura et al. 1987). This finding stresses the importance of proper intratumoral injection to prevent intravascular leakage with, consequently, more severe side-effects. Only sporadic tumor responses have been reported after systemic administration of rHuTNFα in a phase I study (Creagan et al. 1988, Creaven et al. 1989), while no responses have been reported in phase II studies of intravenous rHuTNFα application (Lenk et al. 1989, Schaadt et al. 1990, Kemmeny et al. 1990, Whitehead et al. 1990). Regression of tumor growth and even complete responses were found after intratumoral injection. Pfreundschuh et al. (1989) reported a phase I trial of intratumoral application of rHuTNFα in 21 patients. Complete local response was seen in one patient, partial local response in four patients, minor local response in four patients, and stable disease in two patients. Bartsch et al. (1989) found three partial responses, two minor responses, and three stable diseases in a the total of fourteen injected tumors with rHuTNFα in a phase I study. In our study eight patients showed stable disease. As this antitumor effect could not be related to changes in overall host defence parameters, systemic activation of the immune system by rHuTNFα seems not likely to be of importance. In this study phenotypical parameters of peripheral lymphocytes were measured in five patients at three different dose levels. No significant changes in relative numbers of subpopulations were found. It is not certain whether these phenotypical parameters reflect a functional property of the identified cells. However, it is unlikely that the immunological parameters as measured in peripheral blood samples can reflect local activation or proliferation of tumor infiltrating cells by TNFα, especially when it is given intratumoral. This concept of local activation of tumor infiltrating lymphocytes (TIL) by cytokines has been demonstrated in primary and metastatic liver tumors in an in vitro model (Shimizu et al. 1991). Another mechanism by which rHuTNFα may conduct its antitumor effect is the effect on endothelial cells which results in thrombi formation and finally hemorrhagic necrosis. Kaposi's sarcoma showed tumor regression in 15 out of 16 tumors injected with rHuTNFα (Kahn et al. 1989). Since Kaposi's sarcoma is a neoplastic endothelial cell

proliferation, the effects rHuTNFα has on endothelium may account for the tumor responses seen, although direct cytotoxicity on tumor cells may be of equal importance. It is likely that a critical local rHuTNFα concentration at the tumor site is important for the induction of a tumor response. This critical local TNFα concentration cannot be reached by intravenous administration due to excessive toxicity, and the short half-life of TNFα of approximately 20 minutes. Other mechanisms of improving local concentration of rHuTNFα at the site of malignancy are being investigated, such as liposomes filled with rHuTNFα (Debs et al. 1990), and the genetic engineering of TIL to produce TNFα (Culliton 1990).

In conclusion: this study shows that local injection of rHuTNFα in a liver metastasis by sonographic control is a save procedure and obtains maximal concentration of the drug at the tumor site, while reducing systemic toxicity. The maximal tolerated dose by this route and schedule is higher than 2.31 $x10^6$ U/injection. Although the scope of drug administration by intratumoral injection is limited for the treatment of disseminated malignancies, it may help to elucidate the relevance of rHuTNFα as an anti-neoplastic agent.

CHAPTER 5

DISCUSSION AND CONCLUSIONS OF PART 1

The aim of the studies presented in the first part of this thesis was to assess the potential of TNFα as immunotherapeutic agent in vivo. The results of the experiments demonstrate that administration of exogenous recombinant TNFα to tumor bearing rats can inhibit tumor growth (chapter 2). The efficacy of TNFα treatment on tumor growth depended on the localisation of the tumor and on the way of administration. When colon cancer CC531 was grown under the renal capsule or s.c., TNFα had significant growth inhibitory activity. When tumor CC531 was grown as established tumor in an artificial liver metastases model, TNFα treatment did not inhibit its growth, even not when it was administered in sublethal doses via continuous i.v. infusion. It appeared that local injection of TNFα within a tumor was the most effective way of administration. Administered in this way, TNFα occasionally led to tumor regression. The results obtained in the rats are in agreement with the results obtained in mice. It was shown in mice that TNFα when given i.l. was more efficacious than when administered i.v. (Haranaka et al 1984). The higher concentration of TNFα within a tumor that can be achieved by i.l. administration probably accounts for this phenomenon. The dosis of TNFα that can be administered systemically is limited because of its toxicity (Caput et al. 1986). TNFα is one of the mediators of the septic shock syndrome and provokes the clinical symptoms of the septic shock syndrome when administered in high doses.

The results of the clinical studies do also support the notion that TNFα can best be administered i.l.. Systemic administration of TNFα is poorly efficacious (Blick et al. 1987, Feinberg et al. 1988, Creavan et al. 1989), while i.l. administration allows for much higher doses to be given (chapter 4) and has more therapeutic potential (Bartsch et al. 1989, Pfreundschuh et al. 1989). However, regarding current methods of i.l. administration the number of patients eligible for such a treatment is low. Therefore new techniques must be developed to administer cytokines locally making cytokine-therapy also applicable for patients with disseminated cancer. Such techniques are currently being developed. These include attempts to genetically engineer tumor infiltrating lymphocytes to increase their cytokine production, and attempts to deliver cytokines locally by means of liposomes.

There is, however, another problem with regard to TNFα's potential as

immunotherapeutic agent. As demonstrated in our rats, TNFα monotherapy never resulted in complete cures. In mice complete cures obtained with TNFα therapy have been reported (Haranaka et al. 1984). Asher et al. (1987) have demonstrated that this only accounted for mice with immunogenic tumors. They compared the antitumor effect of TNFα against both immunogenic sarcoma's and non-immunogenic sarcoma's and showed that complete cures could only be accomplished in mice with the immunogenic variants. This is also in agreement with the hypothesis of Havell (1988), concerning TNFα's mechanism of antitumor action. Havell suggested that to obtain complete cures, the effects of TNFα on tumor vasculature as well as the immunomodulatory activities of TNFα were important. We have show that the antitumor activity of TNFα in our rat model is mediated by the immune system. This was demonstrated by the fact that TNF had lost its antitumor effect in rats that were immunosuppressed (chapter 2). Because human tumors are generally non-immunogenic, clinical application of TNFα for curative purposes may be limited to only a few types of tumors. It might lead to tumor growth inhibition of nonimmunogenic human tumors by its action on tumor endothelium but complete irradication of the tumor by TNFα's action is unlikely to happen.

Therefore it is recommended not to treat patients solely with TNFα. Attention should rather be focussed on the use of TNFα in combination with other agents or treatment modalities. Several combinations have already been tested. Synergistic actions of TNFα have been reported when it was applied in combination with interferons (Van der Heyden et al. 1986, Marquet et al. 1987), with interleukin 2, with chemotherapy (Das et al. 1989, Schiller et al. 1990), and with hyperthermia (Klostergaard et al. 1989, Iizumi et al. 1989). Synergistic actions of induced endogenous TNFα with recombinant exogenous TNFα have also been documented (Inugawa et al. 1988). This therapy is known as EET. We have examined whether ABPP was able to induce the release of TNFα in vivo and whether this endogenous TNFα contributed to the antitumor actions of ABPP. It was found that after ABPP treatment endogenous TNFα was significantly increased and that blocking of the effects of TNFα with a polyclonal antiserum could reduce the antitumor effects of ABPP. Because Marquet et al. (1988) have already demonstrated that ABPP acts synergistically with

recombinant TNFα we think that ABPP, or ABPP related compounds, might be worthwile to be used in EET.

In conclusion, TNFα has immunotherapeutic potential. It is, however, unlikely that treatment with TNFα alone can lead to complete erradication of human tumors, due to the fact that human tumors are in general non-immunogenic. Therefore it is recommended to use TNFα only in combination with other biological response modifiers or with other treatment modalities. One suggestion is to combine induced endogenous TNFα with exogenous recombinant TNFα. When it is decided to apply TNFα in the clinic, it can best be administered i.l. This way of administration is clearly more effective than systemic administration.

PART 2

CHAPTER 6

SIGNIFICANCE OF TNFα IN HOST-VERSUS-GRAFT AND GRAFT-VERSUS-HOST REACTIONS; A REVIEW

INTRODUCTION

Rejection of an organ graft is mediated by a complicated immune reaction involving various cell types (Strom 1988, Halloran et al. 1989). The T-cell is thought to be at the centre of the process of acute graft rejection (Bach et al. 1987, Roitt et al. 1989). Briefly stated, the helper T-lymphocyte is thought to recognize class II HLA antigens on the surface of the allogeneic cell. Upon recognition the T-helper cell releases lymphokines that act in an autocrine and paracrine fashion resulting in activation of monocytes/macrophages, T-cells (T-helper cells and T-cytotoxic cells), and polymorphonuclear cells. Other cytokines are also released attracting various immune cells to the graft. Activated cells within the graft finally lead to graft destruction. The significance of cytokines in the proces of allograft rejection has been recognized (Prowse et al. 1985, Hao et al. 1990), especially when it was shown that Cyclosporin A (CsA) acts through inhibition of cytokine production (Cockfield et al. 1991). However, attention has been focussed mainly on the role of interleukin-2 in this process. Less attention has been paid to the involvement of TNFα.
In the next section an overview is presented of the immunomodulatory activities of TNFα and of the significance of these actions with regard to host-versus-graft and graft-versus-host reactions.

IMMUNOMODULATORY ACTIONS OF TNFα

T-cells:

T-cells have been shown to produce TNFα when stimulated with mitogen or with antigen (Sung et al. 1988, Steffen et al. 1988). TNFα is released rapidly after stimulation with allogeneic cells in a mixed lymphocyte culture (MLC), being demonstrable already after 1 hour (Ranges et al. 1987, Shalaby et al. 1988). T-cells become activated thereby increasing the expression of interleukin-2 receptors, TNFα receptors, and of MHC class II antigens (Scheurich et al. 1987, Vink et al. 1990). Addition of TNFα to an MLC results in increased T-cell proliferation, while blocking of TNFα activity inhibits T-cell proliferation (Yokota et al. 1988, Talmadge et al. 1988, Shalaby et al. 1988). Furthermore, TNFα has been demonstrated to induce T-cell cytotoxicity in an MLC, reversing the dose-dependent inhibition of cytotoxic T-

cells by TGFβ (Ranges et al. 1987). In vivo blocking of TNFα activity at the time of antigen priming has been demonstrated to interfere negatively with the generation of both CD4 positive T-cells mediating contact sensitivity, and with CD8 positive T-cells mediating secondary cytotoxic responses (Chavin et al. 1991).
TNFα has also been shown to promote adhesion of lymphocytes to endothelium (Cavender 1987, Issekutz 1990), a first and important step in the migration of lymphocytes out of the blood into the inflammatory site .

B-cells:

TNFα has been shown to posses growth stimulatory actions on B-lymphocytes. This accounts for both normal and transformed B-cells (Digel et al. 1989). In vivo, TNFα has adjuvant activity for antibody production as shown by increased anti-BSA (bovine serum albumin) serum antibodies after immunisation with BSA, followed 48 houres later by TNFα administration. (Talmadge et al. 1988).

Natural Killer (NK)-cells:

Conflicting results with regard to TNFα's action on NK-cells have been published. Ostensen et al. (1987) reported enhancing effects of TNFα on NK-cell activity; Talmadge et al. (1988) found no effect of TNFα on NK-cell activity, while Gordon et al. (1990) reported diminished activity of NK-cells after incubation with TNFα. Consensus does exist as to the synergy of interleukin-2 and TNFα in inducing lymphokine activated killer (LAK)-cell activity. This might be based on TNFα's ability to induce the expression of IL-2 receptors on NK-cells (Ostensen et al. 1987, Owen-Staub et al. 1988).

Mononuclear cells:

Monocytes/macrophages are involved in various immune processes and thought to be important during allograft rejection. On the one hand by acting as antigen presenting cells, and on the other hand by releasing immunomodulating agents (Hori et al. 1989, Kornbluth et al. 1986, Wright et al. 1988, Decker et al. 1989). Among these are interleukin-1 and TNFα, both of major importance in T-cell activation (see section T-

cells). TNFα has autocrine effects on macrophages, activating them to become tumoricidal, partially by the release of reactive nitrogen intermediates and oxygen intermediates (Philip et al. 1986, Heidenreich et al. 1988, Keller et al. 1990). These intermediates might also be partly responsible for the cell destruction often seen in the latter stages of graft rejection. Another important effect of TNFα with regard to the rejection process, is its chemotactic effect on monocytes as shown by the enhanced migration of monocytes through polycarbonate and nitrocellulose filters (Ming et al. 1987).

Polymorphonuclear cells:

TNFα activates neutrophils. Phagocytic activity of neutrophils is enhanced after they are incubated in the presence of TNFα (Klebanof et al. 1986, Shalaby et al. 1985); the cytostatic/cytolytic capacity of neutrophils on tumor cells and fungae is significantly increased by TNFα (Shau 1988, Djeu et al. 1986), and the production and release of reactive oxygen intermediates is increased (Klebanof et al. 1986, Figari et al. 1987). TNFα is chemotactic for neutrophils, not only attracting them to the site of inflammation but also promoting adherence to endothelial cells, and stimulating them to actively pass the endothelial lining (Gamble et al. 1985, Seow et al. 1987, Wankowicz et al. 1988, Mason et al. 1989).

The immunomodulatory actions of TNFα discussed, suggest that TNFα indeed may be an important mediator during graft rejection. It is released upon allogeneic recognition. It stimulates the proliferation of T-cells and the development of cytotoxicity of T-cells. It is chemotactic for T-cells, monocytes and polymorphonuclear cells attracting them to a site of inflammation and at the same time activating them to release other immune mediators. Thereby TNFα, especially in high concentrations, can be cytolytic to cells of the graft. Another feature of TNFα, discussed in chapter 2 of this thesis, is its modulating effect on endothelium increasing the expression of MHC-antigens and various adhesion molecules, and inducing morphological changes, thereby facilitating extravasation of immune cells.

Whether TNFα actually is of significance during host-versus-graft and graft-versus-host can only be shown in models in which TNFα can be demonstrated, either circulating or locally within the graft, and in which the effects of TNFα can be blocked. During the last few years such systems have been studied. The next section gives an overview of the literature that has been published on this subject.

TNFα IN HOST-VERSUS-GRAFT REACTIONS

The first report suggesting an important role of TNFα during graft rejection, appeared in 1987. Maury and Teppo (1987) demonstrated that serum TNFα was increased in patients during rejection crises after kidney transplantation. They also showed that the actual increase of serum TNFα preceded the clinical diagnosis of rejection by one or two days. Noronha et al. (1990) have confirmed these findings showing that TNFα serum levels were not increased in patients with stable grafts, but only in patients that rejected their grafts. Unfortumately, TNFα serum levels are also increased due to other circumstances such as viral infections, making the use of serum TNFα as sole parameter of rejection disputable (Noronha et al. 1990, McLaughlin et al. 1991). By now TNFα has been demonstrated in sera of patients after transplantation of various organs, such as kidney, liver, and bone marrow (Holler et al. 1990, Vogel et al. 1990, Imagawa et al. 1990, Noronha et al. 1990, Hoffmann et al. 1991). In animal models of organ transplantation TNFα has also been demonstrated systemically. Increased serum TNFα levels have been demonstrated in rats after heart transplantation (Imagawa et al. 1990, Hancock et al. 1991). In this model Imagawa et al. (1990) demonstrated that peak-levels of TNFα were reached 3 days before the hearts were rejected according to clinical parameters. Lowry et al. (1988) showed that graft infiltrating cells of the monocyte/macrophage lineage produced spontaneously more TNFα than cells of control animals. Northern blot analysis of cytokine mRNA levels in transplanted rat lungs during rejection crises showed increased levels of TNFα mRNA (Jordan et al. 1991). Finally it was shown that various cytokines, among which TNFα, were produced by infiltrating cells within

sponge matrix allografts (Ford et al. 1990). All these studies thus demonstrate that TNFα is released during the proces of allograft rejection and that a correlation exists between TNFα levels, either systemically or locally, and the severity of the rejection process. In animal models of organ transplantation it has been demonstrated that blocking of TNFα activity via anti-TNFα serum or anti-TNFα monoclonal antibodies can prolong the survival of allografts. It was shown that in rats rejection of heart grafts could be delayed by treatment with rabbit anti-TNFα serum (Imagawa et al. 1990, Seu et al. 1991, see chapter 7). We have shown that treatment with rabbit anti-TNFα serum prolongs the survival of small bowel grafts as well (see chapter 8). It was further demonstrated that anti-TNFα monoclonal antibodies when administered locally, together with anti-interferon gamma monoclonal antibodies could prolong the survival of skin allografts, but not anti-TNFα monoclonal antibodies alone (Stevens et al. 1990). In addition it was shown that administration of TNFα to animals after grafting of allogeneic hearts, shortened graft survival (Imagawa et al. 1990, see chapter 7). These results prove that TNFα not only can be demonstrated in host with rejecting grafts, but that it also plays an important role in mediating the rejection response.

TNFα IN GRAFT-VERSUS-HOST REACTIONS

Another immune phenomenon in which TNFα theoretically might play a significant role is in graft-versus-host disease (GvHD). The introduction of allogeneic T-lymphocytes into an immunoincompetent host is known to lead to a potentially lethal disease, the GvHD, which is associated with lesions of various organs or tissues. In particular, in the acute phase of the disease, the intestinal tract and the epidermis are affected (Slavin et al. 1973, Rappaport et al. 1979, Sale 1984). Treatment with CsA in models of GvHD ameliorates the detrimental effects of GvHD, again suggesting an involvement of cytokines (Goldin et al. 1989).

In man a correlation exists between TNFα serum levels after bone marrow transplantation and the development of acute GvHD (Symington et al. 1990). Only

one animal study has been published concerning TNFα serum levels during acute GvHD. In this study no TNFα could be demonstrated in mice during GvHD induced in a parent to F1 model (Piguet et al. 1987). Blocking of TNFα with rabbit-anti-TNFα serum has been reported to dimish the severity of GvHD or to prevent the development of the GvHD in mice (Piguet et al. 1987, Shalaby et al. 1989). In rats, no effect of blocking of TNFα activity could be demonstrated in a lethal model of GvHD, while it did prevent the development of GvHD seen after small bowel transplantation which is mild and transient (see chapter 9).

CONCLUSION

TNFα is highly active in mediating various immune responses. It is involved in HvG and GvH reactions, which is proven by the fact that blocking of TNFα activity does prolong graft survival and ameliorates the detrimental effects of GvHD.

CHAPTER 7

ANTI-TNFα SERUM PROLONGS HEART ALLOGRAFT SURVIVAL IN RATS

This chapter is a modified version of the article

Anti-tumor necrosis factor alpha serum prolongs heart allograft survival in rats
by Marcel Scheringa, Ron W.F. de Bruin, Hans Jeekel and Richard L. Marquet

Transp. Proc. 23(1): 547-548, 1991

SUMMARY

Various cytokines have been shown to be involved in the process of graft rejection. Here we report that blocking of TNFα activity with anti-TNFα serum prolongs heart allograft survival, while administration of exogenous TNFα shortens heart graft survival in rats. This was shown in a BN→WAG model of heart transplantation. The animals were treated with 0.25 ml of normal rabbit serum (control), o.25 ml of anti-TNFα serum, or with the combination of 0.25 ml anti-TNFα serum and 0.50 ml of anti-IFN serum administered intravenously from the day of transplantation until graft rejection. Mean survival time of control hearts was 8.1 days whereas in anti-TNFα treated animals the grafts survived for 14 days. No additional effects on heart graft survival could be demonstrated by blocking of interferon activity. Administration (i.p.) of 5 μg of rMuTNFα to graft bearing rats shortened graft survival from 8.1 to 7.2 days. We conclude from these experiments that TNFα is an important mediator of graft rejection and that manipulation of TNFα activity changes the survival times of allogeneic heart grafts.

INTRODUCTION

Cytokines play a crucial role in the process of allograft rejection. The role of especially interleukin-2 in this process has been well established. Less attention has been paid to the involvement of TNFα. TNFα serum levels were shown to be increased during allograft rejection (Maury and Teppo 1987, Noronha et al. 1990, Holler et al. 1990, Vogel et al. 1990, Imagawa et al. 1990, Hoffman et al. 1991); it has been demonstrated that anti-TNFα serum can prevent the development of Graft versus Host Disease (GvHD, Piguet et al. 1987, Shalaby et al. 1989). In another study administration of a combination of anti-TNFα and anti-interferon gamma (anti-IFNγ) monoclonal antibodies resulted in prolonged skin graft survival (Stevens et al. 1990).
In this study we studied the significance of TNFα with regard to the proces of heart allograft rejection. We observed both the effects of administration of exogenous TNFα and of blocking of TNFα activity on heart graft survival in a rat model.

MATERIALS AND METHODS

Rats:

WAG ($RT1^u$) and BN ($RT1^n$) male rats were obtained from Harlan-CPB (Austerlitz, The Netherlands). The animals were bred under specific pathogen-free conditions and were between 12 and 20 weeks of age when used in the experiments. The animals were kept under standard laboratory conditions (12 hours light/12 hours dark) and were fed a standard laboratory diet (Hope Farms). WAG rats were used as recipients, and BN rats were used as cardiac allograft donors.

Heart transplantation:

Heterotopic cardiac grafting was performed according to the method of Ono and Lindsey (1969). Briefly, donor hearts were perfused with chilled heparinized PBS and harvested after ligation of the vena cava and the pulmonary veins. Microvascular anastomoses between donor and recipient aorta and between the pulmonary artery and vena cava were performed using 8-0 suture (B. Braun Melsungen AG, Germany). Cold ischemic time was routinely 20 min. Cardiac activity was assessed daily by abdominal palpation. Rejection was defined as the last day of palpable contractions. Loss of graft function within 48 h of transplant was considered a technical failure, but this never occurred.

Antisera:

RaTNFS as well as RaIFNS were prepared in the laboratory. Rabbits were immunized three times, with a two week interval, with rMuTNFα(B) or with recombinant rat IFN together with complete Freund's adjuvans, administered intracutaneously. One week after the last immunization, blood was collected and serum prepared. RaTNFS had a neutralizing capacity of 100,000 U/ml serum in a standard TNF-cytotoxicity assay (L929). In vivo it protected the rats against an otherwise lethal dose of TNFα. RaIFNS had a neutralizing capacity of 110,000 U/ml serum in a standard antiviral assay. In vivo it inhibited the MHC class II inducing effect of 10,000 U IFN on dendritic cells in the heart of BN rats.

Experimental groups:

Experiment 1: Recipient rats were left untreated (Control group) or were treated with 0.25 ml normal rabbit serum (NRS), 0.25 ml RaTNFS (RaTNFS-group), 0.50 ml RaIFNS (RaIFNS-group), or with the combination of 0.25 ml RaTNFS and 0.50 ml RaIFNS (Combination group). The serum was administered intravenously, daily from the day of transplantation until graft rejection.

Experiment 2: Experimental animals received daily 5 μg of rMuTNFα(B)/rat, from the day of transplantation until graft rejection (for details of rMuTNFα see chapter 2). The results were compared to a historical control group that received no treatment.

Graft survival times for the different treatment groups were compared using the Wilcoxon's Rank Sum test.

RESULTS

Results are summarized in table 7.1. RaTNFS treatment prolonged heart graft survival as did the combination of RaTNFS and RaIFNS ($p<0.05$). RaTNFS prolonged graft survival from 8.1 days (Control group) to 14 days. The combination of RaTNFS and RaIFNS did not lead to any additive effect on graft survival. RaIFNS treatment alone did not prolong heart graft survival significantly.

Administration of rMuTNFα(B) to rats after transplantation shortened graft survival significantly ($p<0.05$). Hearts of TNFα-treated animals were rejected in 7.2 days while control hearts were rejected in 8.0 days.

Table 7.1 Survival times of BN hearts transplanted into WAG recipients.

Treatment	Graft survival mean (days)	Graft survival individual (days)
None (Control)	8.1	8, 8, 8, 8, 8, 8, 8, 9
NRS[1]	8.6	8, 8, 8, 9, 10
RaTNFS[2]	14.0#	12, 12, 13*, 14, 15, 18*
RaIFNS[3]	10.6	9, 9, 10, 12, 13
RaTNFS + RaIFNS[4]	10.8#	8, 9, 11, 12, 12, 13
TNFα[5]	7.2#	7, 7, 7, 7, 8

All serum treated animals received the serum i.v. each day from the day of transplantation untill graft rejection. [1] NRS treated animals received 0.25 ml of normal rabbit serum i.v.; [2] RaTNFS treated animals received 0.25 ml of rabbit anti-TNFα serum i.v.; [3] RaIFNS treated animals received o.50 ml of rabbit anti-IFN serum i.v.; [4] These animals received 0.25 ml of rabbit anti-TNFα serum i.v. and 0.50 ml of rabbit anti-IFN serum i.v.

[5] TNFα treatment consisted of i.p. administration of 5 μg rMuTNFα(B) each day from the day of transplantation untill graft rejection. * Animals were treated with the IgG fraction of RaTNFS.

Significantly different from control and NRS treatment ($p<0.05$).

DISCUSSION

This study demonstrates that monotherapy with anti-TNFα antibodies can prolong the survival of heart allografts. Mean prolongation of survival times was 6 days. This is in agreement with the findings of Imagawa et al. (1990) and Seu et al. (1991). They found a prolongation of heart graft survival of 5 days in a comparable heart transplantation model. In this study we only tested the effect of one low dose of anti TNFα serum. Higher doses might be even more effective in prolonging graft survival. The fact that combination treatment with RaIFNS did not result in better graft survival than monotherapy with RaTNFS was somewhat surprising (Stevens et al.

1990). One explanation might be that the amount of RaIFNS administered was simply too low. Another explanation is that in this model IFN is of minor importance. Indirect evidence for this explanation comes from the experiments by IJzermans et al. (1987) who showed that in the WAG to BN combination administration of IFN did not have any effect on heart graft survival.

An important observation made in this study is that graft survival is significantly shortened when exogenous TNFα is administered to rats after transplantation. This phenomenon has also been described by Imagawa et al. (1990). This observation is of significance because it has been demonstrated that TNFα is released upon anti-rejection therapy with anti-CD3 monoclonal antibodies (Debets et al. 1989, Woodle et al. 1991). Treatment with anti-CD3 monoclonal antibodies is especially used during rejection crises. The amount of TNFα released during treatment with such antibodies gives rise to clinical problems like high fever, chills and rigors. Besides being very inconvenient for the patient, these high TNFα levels might have a negative effect on the graft, as suggested by our results. Therefore it is recommended to treat patients with rejecting grafts not only with anti-CD3 antibodies but with such antibodies in conjunction with anti-TNFα antibodies. This might be benificial for the patient by preventing the toxicity in these patients and at the same time by contributing to the anti rejection therapy. It has already been shown in mice that anti-TNFα antibodies indeed can abrogate the side effects of anti-CD3 treatment (Ferran et al. 1991).

In conclusion: this study demonstrates that TNFα is indeed involved in the proces of heart allograft rejection and that survival times can be changed by manipulating the TNFα activity.

CHAPTER 8

ANTI-TNFα ANTIBODIES PROLONG SMALL BOWEL ALLOGRAFT SURVIVAL IN RATS

This chapter is an adapted version of the article

Anti-tumor necrosis factor alpha antibodies prolong
allograft survival in rats
by Marcel Scheringa, Ron W.F. de Bruin, Erik Heineman,
Hans Jeekel and Richard L. Marquet.

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SUMMARY

Blocking of TNFα activity by anti-TNFα antiserum has been shown to prolong the survival of heart allografts. This study was performed to examine whether blocking of TNFα activity prolonged the survival of small bowel allografts as well, and whether TNFα is involved in graft-versus-host disease (GvHD). Control animals received no treatment after small bowel transplantation (SBT), whereas experimental animals recieved 0.50 ml of the IgG fraction of rabbit anti-TNFα serum i.v. daily from the day of transplantation untill graft rejection. It was shown that this treatment prolonged the survival of the small bowel grafts. The experimental animals showed no signs of GvHD after SBT, while 40% of the control animals did. This indicated that TNFα is also involved in this process. Treatment with anti-TNFα serum of rats with a lethal form of GvHD (Parent→F1 model) did not result in abrogation of the GvHD. We conclude from these experiments that blocking of TNFα activity prolongs small bowel allograft survival in rats. Furthermore it is indicated that TNFα is also a mediator of GvHD.

INTRODUCTION

Cytokines play a crucial role in the process of allograft rejection. Among the cytokines of interest is tumor necrosis factor alpha (TNFα), a cytokine originally described as a serum factor with tumor necrotizing ability (Carswell et al. 1975) but now recognized as a cytokine with pleiotropic effects (see chapter 1).

TNFα serum levels were found to be increased during rejection of various human allografts like kidney (Maury and Teppo 1987) and liver (Vogel et al. 1990, Imagawa et al. 1990). In animal models of heart allotransplantation TNFα serum levels have been reported to be increased as well (Imagawa et al. 1990). Blocking of TNFα-activity with poly- or monoclonal antibodies in these models results in prolonged graft survival (see chapter 7, Seu et al. 1991), while administration of exogenous TNFα results in accelerated rejection of the graft (see chapter 7, Imagawa et al. 1990).

Besides a role during graft rejection, TNFα has also been shown to be involved in Graft versus Host Disease (GvHD). TNFα has been shown to be a major effector of

the gut lesions associated with GvHD (Piguet et al. 1987) and anti-TNFα antibodies can prevent the development of GvHD (Shalaby et al. 1989).

Because of these effects of anti-TNFα treatment on both graft survival and GvHD, anti-TNFα treatment may have potential in small bowel transplantation.

Small bowel transplantation would be the therapy of choice for patients with the short bowel syndrome. These patients are now treated with total parenteral nutrition. In the long term, problems such as recurrent catheter sepsis, thrombosis of veins and liver damage will result in morbidity and even mortality of these patients. Clinical small bowel transplantation is, however, still severely hampered by rejection of the graft (Schraut 1988). Occurrence of GvHD remains to be established in patients but in experimental settings, 40% of rats have clinical signs of GvHD after small bowel transplantation (de Bruin et al. 1990).

Therefore this investigation was undertaken to study the effects of polyclonal rabbit-anti-TNFα serum on small bowel allograft survival. Orthotopic fully allogeneic small bowel transplantations were performed, graft survival was measured and occurrence of GvHD was analysed.

We also measured the effects of this antiserum on the survival of rats with a lethal form of GvHD, since the GvHD seen after small bowel transplantation is mild and transient (de Bruin et al. 1990).

MATERIAL AND METHODS

Animals:

WAG ($RT1^u$), BN ($RT1^n$), and (WAGxBN) F1 hybrid male rats were used in the experiments. WAG and BN rats were obtained from Harlan-CPB (Austerlitz, The Netherlands). The animals were bred under specific pathogen-free conditions and were between 12 and 20 weeks of age when used in the experiments. The animals were kept under standard laboratory conditions (12 hours light/12 hours dark) and were fed a standard laboratory diet (Hope Farms).

Small bowel transplantation (SBT):

Animals used as small bowel donors were deprived of food 24 hours before transplantation. Orthotopic total SBT (WAG→BN) was performed as described earlier (Saat et al. 1989). Briefly, the total small bowel, from the ligament of Treitz to the terminal ileum was harvested along with its vascular pedicle, consisting of the portal vein and superior mesenteric artery. In the recipient the aorta and inferior caval vein were clamped and end-to-side anastomoses were performed between the recipient aorta and caval vein and donor superior mesenteric artery and portal vein, respectively. The recipient small bowel was resected and the graft was anastomosed proximally with the host's duodenum and distally with the remaining terminal ileum.
All recipients received subcutaneously a single dose of 20,000 U of penicillin and 20 mg streptomycin (Depomycine, Gist-Brocades, The Netherlands) posttransplant. After transplantation, rats received standard rat chow and water ad libitum. The animals were examined for signs of GvHD; the severity of GvHD was clinically assessed. Three grades were distinguished, as described earlier (Saat et al. 1989). In the present experiments only grade 1 GvHD (light redness of snout, ears and paws) was observed. Death of the animals was considered the endstage of rejection unless they died within 4 days. Then they were considered to be technical failures. After death, autopsy was performed to confirm or exclude rejection, using the criteria by Schraut and Lee (1986). Briefly, acute graft rejection was indicated by signs of peritonitis, a thin-walled distended graft and unimpaired intestinal anastomoses.

Induction of GvHD:

To induce lethal GvHD, (WAGxBN) F1 rats were irradiated with 5 Gy. Thereafter they received intravenously 5 x 10^7 BN spleen cells (Parent→F_1 model). Diagnosis of GvHD was performed according to the appearance of clinical signs (redness of ears, snout and paws, alopecia, hunched posture and progressive weight loss). Weight of the animals was measured and survival times of the animals were recorded.

Rabbit-anti-TNFα serum (RaTNFS):

RaTNFS was prepared in the laboratory (for details see chapter 7). It had a

neutralizing activity of 100,000 U/ml serum in a standard TNFα cytotoxicity assay (L929). The serum was previously shown to be effective in a heart allotransplantation model in which it prolonged survival from 8.1±0.4 (mean survival time ± standard deviation (MST±SD)) days to 14.0±2.3 days (see chapter 7). In the small bowel experiments, rats were treated with the IgG fraction of RaTNFS. Purification was performed by using a protein-A column. The protein concentration of the purified IgG fraction and of the serum IgG was equivalent.

Experimental design:

SBT: Control animals (group 1, n=10) received no treatment after SBT. Experimental animals (group 2, n=7) received daily 0.50 ml of the IgG fraction of rabbit-anti-TNFα serum intravenously for 10 days, starting at the day of transplantation.

GvHD: Control animals were treated with 0.25 ml of normal rabbit serum per day on days 0, 2, 4, 5, 6, 7 and 8 (day 0 = day of GvHD induction, n=5); experimental animals received 0.25 ml of RaTNFS on the same days (n=5).

Statistical analysis:

Group survival was expressed as (MST±SD. Comparison between groups was performed by Wilcoxon's Rank Sum test.

RESULTS

SBT: Treatment with the IgG fraction of RaTNFS resulted in prolonged graft survival ($p<0.05$, Table 8.1). The animals in group 1 survived with an MST±SD of 12.8±3.1 days while the animals in group 2 survived 26±22.9 days. 57% of the animals in group 2 had comparable survival times as the animals in group 1, while 43 % had extended survival times (34, 37 and 70 days, respectively). 40% of the animals in the control group developed a mild, transient grade 1 GvHD. The animals treated with the IgG fraction of RaTNFS showed no signs of GvHD.

GvHD: Treatment with RaTNFS had neither an effect on the time of onset of GvHD, nor on the course of GvHD. GvHD in both groups was diagnosed at day 11±1. Animals in the control group died between day 15 and 20 (MST±SD = 17.4±2.4). Animals in the experimental group died between day 16 and 20 (MST±SD = 17.8±2.0). No difference in mean weights between groups 4 and 5 were measured.

Table 8.1 Survival times of small bowel grafts

Group	MST ± SD[1]	Graft survival individual (days)	% GvHD[2]
Control[4]	13 ± 3	6, 10, 11, 12, 14, 14, 15, 15, 15, 16	40
RaTNFIgG[5]	26 ± 23[3]	8, 9, 10, 14, 34, 37, 70	0

[1]: mean survival time ± standard deviation
[2]: percentage of animals with clinical signs of GvHD
[3]: significantly different from control ($p<0.05$, Wilcoxon's Rank Sum Test)
[4]Control animals received no treatment.
[5]Animals received 0.50 ml of the IgG fraction of RaTNFS daily for 10 days (day 0-10).

DISCUSSION

We reasoned that anti-TNFα treatment might be of importance in the treatment of rejection of small bowel grafts. Firstly, because of its positive effect on the survival of vascularized grafts, an effect which we have reported earlier (see chapter 7). Secondly, because it might have an effect on GvHD (Piguet et al. 1987, Shalaby et al. 1989), a reaction frequently seen in experimental SBT (Schraut 1988, de Bruin et al. 1990).

Indeed treatment with anti-TNFα antibodies resulted in extended graft survival and in abrogation of GvHD in rats with small bowel grafts. None of the animals treated with

anti-TNFα IgG after small bowel transplantation developed clinical GvHD while 40 % of the control animals did. Surprisingly, we were not able to find any benificial effect of anti-TNFα treatment in our lethal GvHD model. This suggests that the ability to suppress GvHD by means of anti-TNFα antibodies is model dependent. GvHD after small bowel transplantation is mild, transient and lasts only for about 3 days (this study, de Bruin et al. 1990). Obviously a mild, transient GvHD is easier to suppress than the vigorous lethal GvHD in the parent→F_1 model.

We showed earlier that the GvHD apparent after small bowel transplantation can be abrogated by donor pretreatment with 5 or 10 Gy (Saat et al. 1989). However, the absence of GvHD resulted in shortening of graft survival. Anti-TNFα treatment is clearly superior to irradiation in that it both suppresses clinical GvHD and at the same time prolongs graft survival.

We think that the results presented in this study are promising with respect to therapy of rejection crises after small bowel transplantation. Combining anti-cytokine therapies with for example CsA or FK506 could be an effective rejection treatment modality.

In summary: our results indicate that blocking of TNFα results in prolonged small bowel allograft survival and in abrogation of the GvHD seen in this fully allogeneic transplantation model.

CHAPTER 9

DISCUSSION AND CONCLUSIONS OF PART 2

The aim of the studies described in the second part of this thesis was to assess the significance of TNFα with regard to graft rejection. The involvement of TNFα in allograft rejection was suggested by the observation that TNFα serum levels were increased during rejection of various organ grafts in man (Maury et al 1987, Noronha et al 1990, Holler et al 1990, Vogel et al 1990, Hoffmann et al 1990, Imagawa et al 1990). The first report on this subject came from Maury and Teppo in 1987. They showed increased TNFα serum levels in patients with rejecting kidney allografts. Direct evidence to support this suggestion was up to now not available. The results of the experiments described in chapter 6 and chapter 7 are among the first to prove the involvement of TNFα in the rejection process directly. It was shown that blocking of endogenous TNFα activity could prolong the survival of heart allografts as well as small bowel allografts in rats. These results are in agreement with the observations made by Imagawa et al (1990) who showed that during rejection of heart allografts in rats the TNFα serum levels were increased and that blocking of TNFα activity prolonged graft survival.

This knowledge could have clinical implications. Anti-human TNFα monoclonal antibodies are commercially available and can therefore be applied in patients. It is, however, obvious that a complicated process such as rejection of allografts, can not be abrogated by blocking of one single mediator of the process. We therefore suggest to use anti-TNFα therapy in combination with other immunosuppressive modalities and to evaluate whether it has any additional effects.

A potential difficulty in applying anti-TNFα monoclonal antibodies for patients with organ grafts, is the development of anti-xenotypic or anti-idiotypic antibodies. Forming of anti-xenotypic antibodies can be prevented by humanizing of antibodies but anti-idiotypic antibodies will eventually be produced. An alternative method of anti-TNFα therapy would be the use of soluble TNFα-receptors. Theoretically TNFα activity could be blocked chronically in this way without incurring mayor problems. It has already been demonstrated that blocking of interleukin 1 with its soluble receptor can prolong graft survival. Unfortunately the gene for the TNFα receptor has as yet not been isolated and it is therefore impossible to obtain reliable amounts of the soluble form of the receptor for clinical use.

A significant observation presented in chapter 7 is that administration of exogenous recombinant TNFα to rats with a heart allograft shortens graft survival. It is known that during certain immunosuppressive treatments in man, i.e. treatment with anti-CD3 antibodies, TNFα serum/plasma levels rice significantly (Woodle et al 1991), especially during the first time these antibodies are administered (Debets et al 1989). This might have a negative effect on the graft. Therefore we suggest to administer anti-TNFα antibodies to patients when they are treated with anti-CD3 antibodies. This will reduce the side effects that are observed during such therapy (Ferran et al 1991) and at the same time increase the immunosuppressive effect.
The results of the few experiments that have been performed regarding the effects of TNFα during GvH reactions (chapter 8) indicate that TNFα is also of significance in this reaction. We showed that anti-TNFα treatment had no effect on the survival of rats with lethal GvHD, but that it abrogated the mild form of GvHD seen after SBT. Studies in mice show that blocking of TNFα activity can diminish the severity or prevent the development of GvHD (Piguet et al 1987, Shalaby et al 1989). It appearred, however, that in a parent to F1 model of GvHD in mice no TNFα could be demonstrated in the serum (Piguet et al 1987).

In conclusion, TNFα is an important mediator of graft rejection. Blocking of its activity prolongs graft survival in rats, while administration of TNFα to rats with organ grafts shortens graft survival. Studies performed to assess the significance of TNFα in GvH reactions indicate that it also might be an important mediator of this process.

SUMMARY, SAMENVATTING

SUMMARY

The immune system uses an ingenious communication systen in order to fulfill its complex tasks. Communication is performed in various ways, one of them by the agency of soluble mediators, generally termed cytokines. From a clinical point of view it is of great interest to analyse the effects of these cytokines. Manipulation of their release or actions might offer new perspectives for clinical entities in which stimulation or suppression of the immune system is essential, such as in cancer immunotherapy and in organ transplantation. In this thesis the effects of the cytokine TNFα on tumor growth and its significance for transplant rejection was studied.

Chapter 1 presents a review on TNFα. Several aspects of TNFα are being discussed varying from the molecular biology to clinical studies. Integrated in this review is the knowledge obtained by the experimental work described in the other chapters of this thesis.

In chapter 2 the effects of TNFα on growth of the rat coloncarcinoma CC531 are presented. It was demonstrated that intralesional administration of TNFα was more effective than systemic administration. TNFα inhibited the growth of tumor CC531 in vivo but it displayed no antiproliferative effects on growth of this tumor in vitro, suggesting that TNFα acted indirectly in vivo through one or more host factors. It was shown that blocking of the immune system via cyclosporin-A abrogated the tumor growth inhibiting effects of intralesional administered TNFα in vivo. In the used rat model the effects of TNFα on tumor vasculature seemed to be of minor importance since the number of vessels per tumor area was the same in treated and untreated animals and since pretreatment with heparin did not influence TNFα's antitumor action. TNFα treatment never resulted in complete cures, therefore it was suggested to apply TNFα in immunotherapy of cancer only in combination with other agents or treatment modalities.

In chapter 3 antitumor effects of induced endogenous TNFα are described. It appeared that the pyrimidinone ABPP could induce the release of TNFα in rats and that this contributed to the tumor growth inhibiting effect of ABPP. This was demonstrated by the fact that blocking of endogenous TNFα activity with anti-TNFα

serum reduced the antiproliferative effects of ABPP. It was concluded that ABPP or ABPP-related compounds might be worthwhile to be used in endogenous-exogenous therapy with TNFα, a therapy already shown to have antitumor potential.

In chapter 4, a phase 1 study with TNFα is presented. Patients with liver metastases, refractory to standard therapy were entered into this study. They received rHuTNFα intralesionally by ultrasound-guided punction. It appeared that these patients tolerated doses up to 350 ug well. This further supports the notion that far more TNFα can be administered locally than systemically. In chapter 5 the results of chapters 2-4 are discussed.

In the second part of this thesis experiments are described concerning TNFα involvement in HvG and GvH reactions. In chapter 6 a review is presented of the immunomodulatory actions of TNFα with emphasis on the relevance of these actions for HvG and GvH reactions.

Prolongation of graft survival by blocking of TNFα activity is demonstrated in chapter 7. It appeared that blocking of TNFα activity with a polyclonal anti-TNFα serum prolonged allogeneic heart graft survival in rats, while administration of recombinant TNFα shortened heart graft survival. It was further shown that blocking of TNFα activity not only prolonged heart graft survival but small bowel allograft survival as well.

The significance of TNFα for GvH reactions was studied (chapter 8). The results of these studies indicated that TNFα is an important mediator of this reaction as well, but further studies need to be performed to gain insight into this reaction. In the final chapter the significance of the findings obtained in chapters 7 and 8 are discussed. It is suggested to start to use anti-TNFα therapy in combination with other immunosuppressive therapies to see whether additional effects can be found. Furthermore it is suggested to use anti-TNFα therapy in combination with anti-CD3 therapy in the clinic, because it is well known that anti-CD3 antibodies can induce the release of TNFα which might be harmfull to the graft, as was shown in chapter 7.

SAMENVATTING

Gedurende de laatste jaren is er veel onderzoek verricht naar de werking van het immuunsysteem. Uit dit onderzoek is naar voren gekomen dat interne en externe kommunikatie van essentieel belang is voor een goed funktioneren van het immuunsysteem. Bij de kommunikatie wordt onder andere gebruik gemaakt van cytokines. Kennis van de aard van deze cytokines en hun werking is onder andere van klinisch belang. Bij immunotherapie van kanker zou gebruik gemaakt kunnen worden van cytokines om het afweersysteem te aktiveren, terwijl inhibitie van het afweersysteem door manipulatie met cytokines van belang zou kunnen zijn voor patienten met een orgaantransplantaat. In deze dissertatie wordt een analyse gemaakt van de mogelijkheden om de cytokine tumor necrose faktor-alfa (TNFα) aan te wenden als immunotherapeuticum voor kanker en van het belang van deze mediator bij transplantaatafstoting.

Het eerste hoofdstuk van de dissertatie geeft een overzicht van de huidige kennis van TNFα. In dit overzicht zijn de resultaten van de overige hoofdstukken reeds verwerkt. In het tweede hoofdstuk zijn experimenten beschreven die tot doel hadden de effekten van TNFα op tumor groei duidelijk te maken. Voor deze experimenten werd gebruik gemaakt van de colontumor CC531, een chemisch geinduceerde ratte-tumor. Het bleek dat TNFα de groei van deze tumor kon remmen wanneer deze onderhuids groeide of onder het nierkapsel, maar niet wanneer de tumor als gevestigde lever metastase groeide. Zelfs kontinue intraveneuze toediening van subletale hoeveelheden TNFα had dan geen effekt op de tumor groei. Lokale injektie van TNFα in de tumor bleek het meest effektief. Omdat TNFα in vitro geen antiproliferatief effekt vertoonde ten aanzien van tumor CC531, werd gepostuleerd dat de werking van TNFα in vivo gemedieerd werd via een komponent van de gastheer. Wanneer het immuuunsysteem van de ratten onderdrukt werd door toediening van het middel cyclosporine-A, had TNFα geen effekt meer op de groei van tumor CC531. Remming van het stollingssyteem, door middel van heparine vlak voor toediening van TNFα, had geen konsekwenties voor het tumor groei remmende effekt van TNFα. Gekonkludeerd wordt dat het antiproliferatieve effekt van TNFα in

deze ratten gemedieerd wordt via hun immuunsysteem. Behandeling van de ratten met TNFα resulteerde nooit in het volledig verdwijnen van de tumor. Daarom werd onderzocht wat de immunotherapeutische potentie van TNFα is wanneer het in kombinatie met andere stoffen wordt toegediend.

Een van de mogelijkheden is om exogeen toegediend TNFα te kombineren met geinduceerd endogeen TNFα. In hoofdstuk 3 werd daarom onderzocht wat de potentie was van de stof ABPP als induktor van endogeen TNFα en of geinduceerd endogeen TNFα inderdaad tumorgroei inhiberend werkte. Dit werd onderzocht in een rattemodel. Het bleek dat na toediening van 200 mg/kg ABPP op twee opeenvolgende dagen de serumspiegels van TNFα in de behandelde ratten signifikant hoger waren dan in de kontrole ratten. Verder bleek dat wanneer de aktiviteit van het endogene TNFα werd geblokkeerd, het antitumor effekt van ABPP met ongeveer 40% verlaagd was. Hieruit werd gekonkludeerd dat het geinduceerde endogene TNFα inderdaad antitumor effekten bezat en dat stoffen zoals ABPP gebruikt zouden kunnen worden bij endogene-exogene therapie met TNFα.

In hoofdstuk 4 zijn de resultaten beschreven van een klinische fase-1 studie. Patiënten met lever metastasen die niet (meer) reageerden op standaard therapie werden op vrijwillige basis tot de studie toegelaten. Zij kregen door middel van echo-geleidde punktie TNFα intralesionaal toegediend. Het bleek dat doseringen tot 350 ug per patient goed werden verdragen. Dit ondersteunde de observatie gedaan in hoofdstuk 2 dat via intralesionale toediening meer TNFα kon worden toegediend. Uit andere klinische studies was inmiddels gebleken dat bij systemische toediening lagere maximale doses werden bereikt.

Een diskussie van de resultaten van de eerste 4 hoofdstukken is gepresenteerd in hoofdstuk 5. De belangrijkste konklusies uit deze hoofdstukken zijn dat TNFα voldoende potentie heeft om aangewend te worden als immunotherapeuticum bij kanker, maar dat het aan te bevelen is om deze mediator alleen in kombinatie met andere agentia te gebruiken. Dit omdat gebleken is uit de gepresenteerde studies en uit de literatuur dat monotherapie met TNFα zelden leidt tot volledige genezing. Verder werd gekonkludeerd dat intralesionale toediening van TNFα een betere antitumor effektiviteit bezit dan systemische toediening.

In de hoofdstukken 6 tot en met 8 is een analyse gemaakt van het belang van TNFα bij de afstoting van organen na transplantatie. In hoofdstuk 6 is een overzicht gepresenteerd van de immunomodulerende effekten van TNFα waarbij de nadruk is gelegd op het belang daarvan bij het afstotingsproces en bij de "graft-versus-host" reaktie. Uit de resultaten van de experimenten gepresenteerd in hoofdstuk 7 bleek dat blokkering van de aktiviteit van TNFα resulteerde in verlenging van harttransplantaat overleving in ratten. Dat dit fenomeen niet orgaan specifiek is bleek uit hoofdstuk 8 waar aangetoond werd dat ook dunne darm transplantaten vertraagd werden afgestoten na blokkering van TNFα aktiviteit. Tevens werd een indikatie verkregen van het belang van TNFα voor de "graft-versus-host" reaktie. Blokkering van TNFα aktiviteit had geen effekt op het ontstaan van "graft-versus-host" verschijnselen in een lethaal model van de "graft-versus-host" ziekte. Geen van de met anti-TNFα behandelde ratten met een dunne darm transplantaat ontwikkelde klinische verschijnselen van "graft-versus-host" ziekte terwijl 40% van de kontrole ratten dit wel deden. Dit is een sterke aanwijzing dat TNFα in ieder geval een mediator is van milde vormen van de "graft-versus-host" ziekte.
Uit de resultaten van hoofdstuk 7 werd tevens duidelijk dat toediening van exogeen TNFα aan ratten met een harttransplantaat resulteerde in een versnelde rejektie.
In hoofdstuk 9 worden de resultaten, beschreven in de hoofdstukken 6 tot en met 8, bediskussieerd en de daaruit voort vloeiende konklusies gepresenteerd. De belangrijkste konklusie is dat TNFα inderdaad een belangrijke mediator is tijdens rejektie van orgaan transplantaten. Tevens dat blokkering van de aktiviteit van TNFα resulteert in verlengde transplantaat overleving en dat toediening van exogeen TNFα de afstoting versnelt. Gesuggereerd wordt om gebruik te maken van deze kennis in de kliniek. Zo is bijvoorbeeld bekend dat tijdens behandeling van transplantatie patiënten met een antilichaam gericht tegen het CD3 antigeen, de spiegels van TNFα in het bloed sterk stijgen. Dit is niet alleen nadelig voor de patient omdat hij/zij daarvan ziek wordt, maar deze hoge endogene TNFα spiegels zouden tevens een negatief effekt op het transplantaat kunnen hebben. Een suggestie is om patiënten die behandeld worden met anti-CD3 antilichamen ook anti-TNFα antilichamen te geven. In muizen is al gebleken dat dit de bijverschijnselen van anti-CD3 behandeling

voorkomt terwijl er tevens van mag worden uitgegaan dat dit additionele waarde heeft wat betreft de immunosuppressie. Verder raden wij aan om de potentie van anti-TNFα antilichamen nader te onderzoeken. Wat betreft de rol van TNFα tijdens de "graft-verus-host" ziekte, achten wij nader onderzoek noodzakelijk om harde konklusies te kunnen trekken.

De eindkonklusie van deze dissertatie is dat de cytokine TNFα onder bepaalde voorwaarden inderdaad potentie heeft als immunotherapeuticum, en tevens dat deze cytokine een belangrijke mediator is van de rejektie van organen na transplantatie.

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LIST OF ABBREVIATIONS

ABPP	bropirimine
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	copy DNA
CsA	cyclosporin-A
CT	chemotherapy
EET	endogenous-exogenous therapy
ELAM	endothelial leukocyte adhesion molecule
GvHD	graft-versus-host disease
HBSS	Hanks ballanced salt solution
HLA	human leukocyte antigen
HvG	host-versus-graft
ICAM	intercellular adhesion molecule
IFN	interferon
IgG	immunoglobulin-G
i.l.	intralesional
IL	interleukin
i.m.	intramuscular
inj.	injection
i.p.	intraperitoneal
IRMA	immuno radiometric assay
i.v.	intravenous
LAK	lymphokine activated killer
LPS	lipopolysaccharide, endotoxin
MHC	major histocompatibility complex
MLC	mixed lymphocyte culture
MST	mean survival time
MTD	maximum tolerated dose
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide
n	number of observations
NK	natural killer
NRS	normal rabbit serum
NSAID	non-steroid anti-inflammatory drug
p	level of significance
PBS	phosphate buffered saline
RaIFNS	rabbit anti-IFN serum
RaTNFS	rabbit anti-TNFα serum
RBC	red blood cell
rHuTNFα	recombinant human TNFα
rMuTNFα	recombinant murine TNFα
RT	radiotherapy
SBT	small bowel transplantation

s.c.	subcutaneous
SD	standard deviation
Sd	stable disease
SRCA	sub-renal capsule assay
TIL	tumor infiltrating lymphocytes
TNFα	tumor necrosis factor-alpha
TNFβ	tumor necrosis factor-beta, lymphotoxin
U	unit
WBC	white blood cell
WHO	World Health Organisation

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Curriculum vitae

De auteur van dit proefschrift werd geboren op 5 december 1963 te Hoogkerk. Het VWO-diploma (Atheneum B) werd behaald in 1982 aan de Gereformeerde Scholengemeenschap te Amersfoort. In datzelfde jaar werd de studie biologie begonnen aan de Biologische Faculteit van de Rijksuniversiteit Groningen. Als hoofdrichting werd gekozen de medisch-biologische. Gedurende een jaar verrichtte hij onderzoek naar het maternaal metabolisme tijdens zwangerschap en lactatie. Dit onderzoek werd uitgevoerd op de afdeling Obstetrie en Gynaecologie van het Akademisch Ziekenhuis te Groningen. In 1987 behaalde hij het doctoraalexamen. Vanaf februari 1988 tot op heden is hij werkzaam als Assistent in Opleiding verbonden aan de afdeling Algemene Heelkunde (hoofd Prof. dr. J. Jeekel) van de Medische Faculteit van de Erasmus Universiteit te Rotterdam.