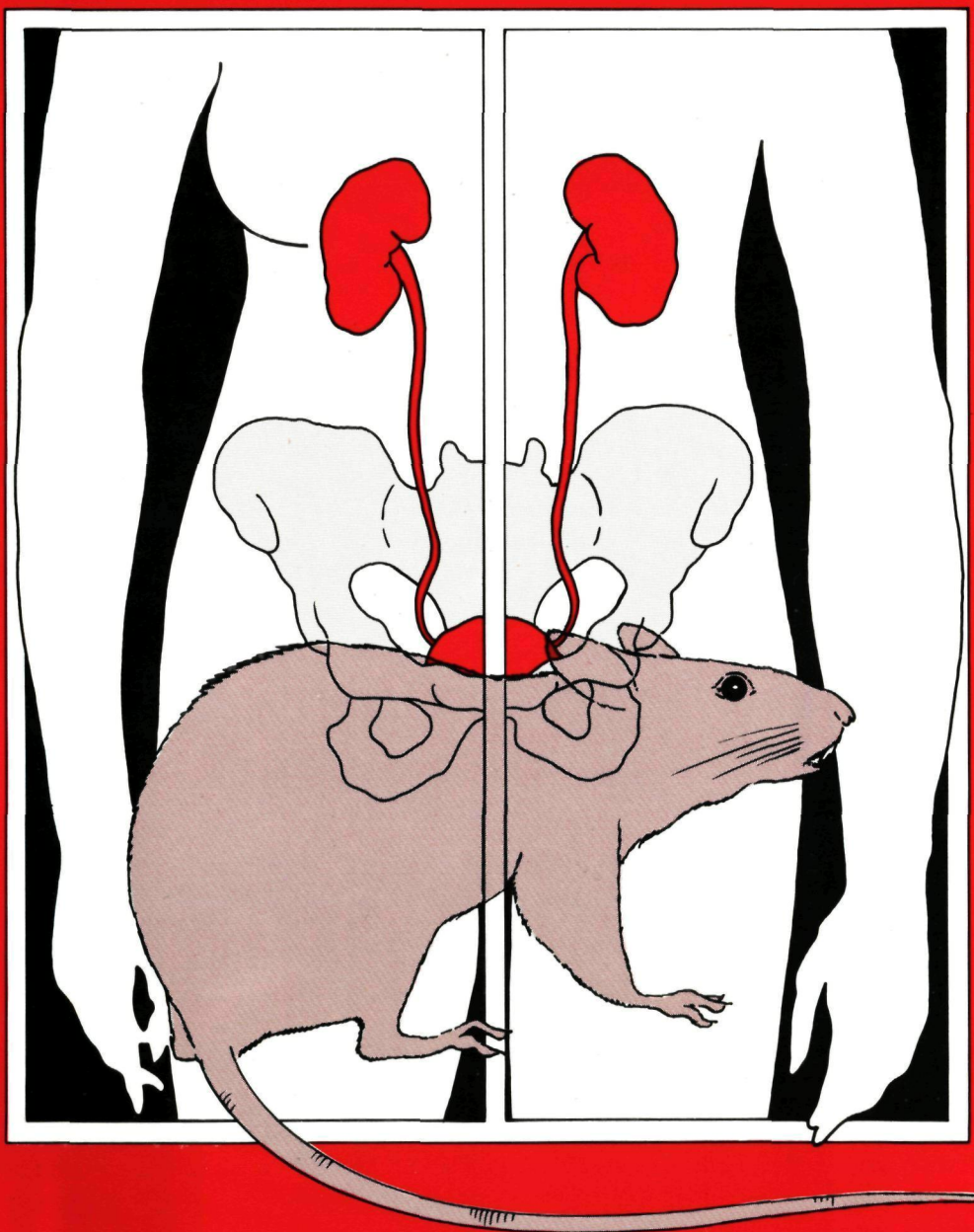
CYTOKINE THERAPY IN EXPERIMENTAL UROLOGICAL CANCER



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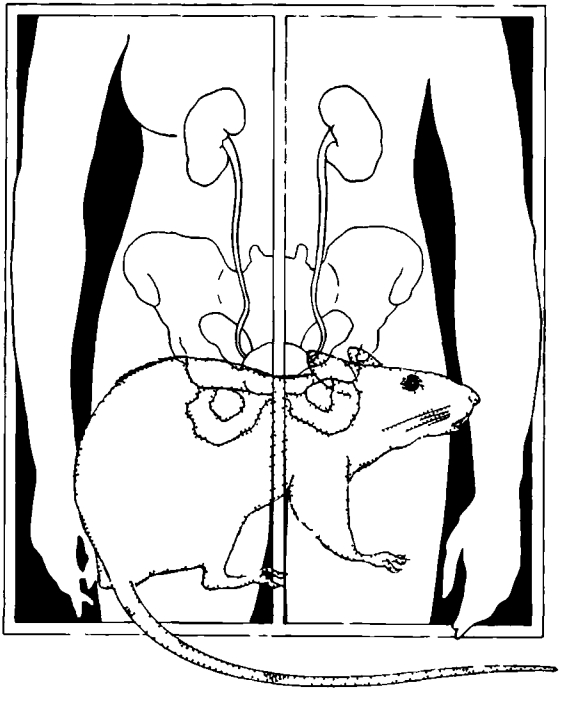

R.J.A. VAN MOORSELAAR

CYTOKINE THERAPY IN EXPERIMENTAL UROLOGICAL CANCER

Een wetenschappelijke proeve op het gebied van de medische wetenschappen,
in het bijzonder de Geneeskunde

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN,
VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN
IN HET OPENBAAR TE VERDEDIGEN OP
DONDERDAG 12 SEPTEMBER 1991
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CONTENTS

"The true remedy for all diseases is Nature's remedy. Nature and Science are at one, believe me; though we are taught differently. Nature has provided, in the white corpuscles as you call them - in the phagocytes as we call them - a natural means of devouring and destroying all disease germs. There is at bottom only one genuinely scientific treatment for all diseases, and that is to stimulate the phagocytes. Stimulate the phagocytes. Drugs are a delusion. Find the germ of the diseqse; prepare from it a suitable antitoxin; inject it three times a day quarter of an hour before meals; and what is the result? The phagocytes are stimulated; they devour the disease; and the patient recovers - unless, of course he's too far gone."

Sir Ralph Bloomfield Bonington in
"The Doctor's Dilemma" by Bernard Shaw, 1906

Ter nagedachtenis van mijn grootmoeder
Aan mijn ouders en grootvader

Voor Jacqueline

CHAPTER I

GENERAL INTRODUCTION

Based on:

The use of animal models in diagnosis and

treatment of renal cell carcinoma:

an overview

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1 ANIMAL MODELS FOR UROLOGICAL CANCERS

The ultimate evaluation of any new therapeutic modality requires careful randomized and controlled clinical studies. Clearly, to define all therapeutic parameters and principles in human studies would be expensive, time consuming, and could involve ethical limitations (1). On the other hand, no single animal model can mimic the human situation or give final answers; it can only provide approximations. Animal models representative for human cancer, however, have proven to be useful to test new therapeutic concepts. In this section an overview will be given of the available animal model systems for urological cancers.

Animal models offer many advantages, e.g. an unlimited number of cells and tumors is available to perform multiple and duplo experiments. Tumor lines can be exchanged among several laboratories, thereby allowing the study of identical material (2).

In general, animal model studies should comply with the following factors: 1) uniformity of genetic strain and age of host, 2) controlled onset of tumor growth, 3) uniformity of tumor type, 4) precise knowledge of tumor load and growth rates, 5) regulation of dietary elements and physical stress, 6) total compliance to drug intake and timing, 7) complete follow-up, 8) uniform control over onset and termination of the study and subject, with all subjects available for autopsy, 9) large numbers in each study group, all monitored at the same time, and 10) one observer making all evaluations (1).

More specifically, an ideal model for renal cell carcinoma should arise spontaneously, and have the histologic characteristics of a pure adenocarcinoma, a predictable growth rate, the capacity to metastasize and the ability to grow without hormonal dependence (3).

Coffey and Isaacs have defined the requirements for an idealized animal model of prostatic cancer (1,4): it should be 1) spontaneous in origin, 2) developed in aged animals, 3) of proven prostatic origin, 4) a slow-growing adenocarcinoma, 5) histologically similar to human prostatic cancer, 6) having a biochemical profile similar to prostate, 7) a malignant phenotype with metastatic pattern to bone and lymph nodes, 8) capable of elevating serum tumor markers, 9) hormone-sensitive, 10) capable of responding to hormonal therapy followed by relapse to hormone-insensitive state, 11) developed and transplantable or easily inducible in syngeneic animals, 12) available in large number of animals for statistical considerations, 13) responsive similar to known human prostatic cancer therapies, 14) available in a wide diversity of tumor types (differentiation, hormonal response, etc.) corresponding to human tumor variability.

Specific requirements for an animal model for bladder cancer include that it should be spontaneously arisen, consist of a pure transitional cell carcinoma, and transplantable in syngeneic animals, subcutaneously as well as intravesically.

In general, animal model systems can be divided into different categories: drug induced tumors, virus-induced tumors and spontaneous tumors (5). Heterotransplanted human tumors in immune-deprived animals form a fourth category.

1.1 Drug-induced tumors

1.1.1 Renal tumors

Both steroid and stilbene estrogens are potent carcinogenic agents in the Syrian golden hamster, malignant renal neoplasms develop after chronic hormone treatment (6,7). These tumors display hormone-dependent growth, and hormonal treatment reduces tumor growth; this degree of hormonal dependence, however, is not found in human renal cancer. Renal adenocarcinomas have been induced by chemical agents including, dimethylnitrosamine (DMN), Formic acid 2-[4-(5-Nitro-2-furyl)-2-thiazolyl]-hydrazide (FNT), and 4'-fluoro-4-biphenylacetamide (FBPA) in different rat strains (8,9,10). Mice show a strain-related susceptibility to renal carcinogenesis by DMN, which relates to the ability of renal microsomes to generate mutagenic metabolites from DMN (11). Heavy metals, like lead, will induce renal tumors in rats and mice, also radiation can induce renal tumors, and the combination of radiation and dietary lead increases the tumor incidence (12,13). A review of chemicals inducing kidney tumors is provided in references 6 and 13.

1.1.2 Prostatic tumors

Prostatic adenocarcinomas can be hormonally induced in Noble (Nb) rats. The Nb rat is an inbred strain developed by R.L. Noble of the Cancer Research Center in Vancouver (14,15). The origin of this strain is not entirely clear, but it probably arose from a Long-Evans rat. The Nb rats have a low incidence of spontaneous prostatic adenocarcinomas, 2 tumors were found in 409 rats older than 13 months (incidence of 0.48%). After prolonged treatment (> 6 months) with s.c. testosterone implants, in approximately 20% of the rats prostatic tumors appear. If testosterone is combined with estrone implants, carcinomas of the prostate occur after a shorter interval, 43 weeks in stead of 59-64 weeks (16). Furthermore, metastatic disease develops in more than 40% of the animals, in which a tumor was induced. All tumors and metastases are readily transplantable in syngeneic animals. In this way the Noble series of prostatic cancers were established (17,18). The different transplantable tumors can be classified as androgen-dependent, autonomous or estrogen-dependent (14). From the more than 200 rat prostatic adenocarcinomas described by Dr. Noble, Dr. Drago choose to study eight tumors (14). These autonomous and androgen-stimulated tumors have been used for extensive chemotherapeutic studies in Nb rats as well as xenografts in nude mice (15,19,20).

Hormonal inducibility of prostatic cancer may be affected by the strain of

rats used. In a direct comparison of two rat strains it was shown that s.c. implantation of silastic tubes with testosterone increases the incidence of microscopic prostate tumors from 10 to 40% in Lobund-Wistar rats (21). In contrast, testosterone did not increase the incidence of these tumors in Sprague-Dawley rats (21). Hildebrand et al. demonstrated differences in decrease of ventral prostate weight between Lewis and Sprague-Dawley rats by the combination of dihydrotestosterone and estradiol (22). Furthermore, age of the rats and absence or presence of testes were important factors that influenced the response of rat prostates to hormonal manipulation (22).

Intravenous administration of the chemical carcinogen 7,12-dimethylbenz(a)-anthracene (DMBA), induces prostatic tumors in castrated rats, mice and hamsters (23). 32% of F344 rats exposed to 3,2"-dimethyl-4-amino-biphenyl (DMABP) develop microscopic prostatic carcinoma, among tumors in several other organs (24). The number of rats with prostatic carcinoma in situ increased to 86%, when the rats were subjected to an intermittent ethinyl estradiol-supplemented diet prior to an injection of DMABP (25). More than 50% of Lobund-Wistar rats develop large, palpable prostatic adenocarcinomas after one i.v. inoculation of N-nitroso-methylurea (MNU), followed 7 days later by a s.c. depot of testosterone (26). Most of the tumor-bearing rats manifested metastases (26). Pour and Stepan were able to produce prostatic tumors in Wistar derived MRC rats with N-nitroso(2-oxopropyl)amine (BOP) in intact and castrated rats during androgen-enhanced cell proliferation in the prostate (27). Testosterone given for life following BOP administration induced prostatic cancer in over 60% of all rats, whereas tumor incidence was significantly lower in rats treated with testosterone for only a short period of time. Simultaneously papillomas and carcinomas of the urinary bladder were induced (27).

Bosland et al. applied temporary chemical castration to Wistar rats, by administration of cyproterone acetate (CA) for 18 days. After three daily testosterone injections following cessation of the chemical castration, a single dose of a carcinogen was given to the rats (28,29). This protocol ensured an intact status of the animals during promotion and progression of the tumors. MNU induced a 30% incidence of carcinomas of the dorsolateral prostate, 63 weeks after MNU injection. The incidence after DMBA administration was lower, only 5%. No carcinomas were found in animals that received MNU or DMBA directly after pretreatment with CA only. In the case of DMABP, however, tumors were not found in rats pretreated with CA and testosterone, but only in animals given CA alone. This effect has been related to the slow metabolization of DMABP to its active form. The transient phase of maximally stimulated cell proliferation in the dorsolateral prostate occurs later in the group receiving CA alone, when compared to the group receiving CA plus testosterone, and can therefore possibly coincide with the delayed formation of activated DAMBP.

The etiology of prostatic cancer is essentially unknown (30). Animal models of induced prostatic tumors can greatly contribute to the identification of agents or factors that modify or induce the carcinogenic process. For immunotherapeutical studies, however, spontaneous tumor models are more useful (see section 1.5).

1.1.3 Bladder tumors

In a number of animal species, like mice, rats, hamsters, guinea pigs, dogs and cows different carcinogens have shown to induce bladder tumors (31-35).

Butyl-(4-hydroxybutyl)nitrosamine (BBN) is potent carcinogen which has been found to induce bladder tumors in mice and dogs (36,37). Rats fed BBN containing drinking water develop, after a latency period of 20 weeks, bladder tumors. Kunze and Schauer added BBN at a dose of 20 mg/kg/day in the drinking water of Wistar rats for a period of 40 to 150 days (38). 88.8% of the rats developed transitional cell carcinoma, whereas 7.9% had squamous cell carcinoma. In a study of Herman et al. 300 ACI rats received 0.05% BBN in the drinking water for 12 weeks, starting at the 7th post natal week (39). Using monoclonal antibodies against cytokeratin 10, they showed development of squamous metaplasia 20 weeks after termination of BBN administration. These lesions progressed to tumors predominantly composed of squamous elements (39). Several permanent cell lines have been established from BBN induced tumors, such as the rat cell lines, NBT-II and BC-50 (40,41). BBN3 is a cell line established in C57BL/6J mice and FCB is derived from a dibutylnitrosamine (DBN) induced anaplastic transitional cell tumor in IFxC57F1 hybrid mice (42,43). Biochemical analyses showed an increase of ras and myc oncogene expression in BBN induced rat bladder tumors, which has been suggested as an early genetic event during bladder carcinogenesis (44,45). Further alteration of these two genes or mutation of additional genes may be required for completion of the malignant transformation (45).

The nitrosamide, N-methyl-N-nitrosurea (MNU), produces tumors in a wide variety of organs, except bladder, if given orally, intravenously or intraperitoneally (46). Hicks and Wakefield induced bladder carcinomas in female Wistar rats by 4 biweekly intravesically applied doses of 1.5 mg MNU. In most animals transitional cell carcinoma developed along with squamous cell carcinoma of the bladder (46). A subcarcinogenic MNU dose had synergistic effects on bladder carcinogenesis in combination with saccharin- or cyclamate containing diets (47). MNU also induced morphological changes in vitro, on normal rat bladder cells and MNU-induced rat bladder tumor cells (48,49). Steinberg et al. used the same MNU treatment schedule as Hicks and Wakefield (46) in female F344 and Copenhagen rats (50). F344 rats developed TCC, no squamous cell or mixed squamous cell/transitional cell carcinomas of the bladder were found, while 50% of the Copenhagen rats developed either squamous cell carcinoma alone or in combination with transitional cell carcinoma. Cytogenetic analyses of the six F344 rat tumors revealed normal

karyotypes in 3 tumors, while karyotypes of the 3 other tumors contained one or more structural markers (50). Biochemically no increase in ras-oncogene expression was detected (50).

Rats, mice and dogs are susceptible to the highly specific carcinogenic action of N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) on the urothelium (51-56). Sprague-Dawley and F344 rats received 0.188% FANFT mixed with the diet (51-53). After three weeks the rats developed hyperplasia of the bladder mucosa, which progresses to carcinomas. After 8 weeks, however, concurrent squamous metaplasia was found and after 11 weeks mixed transitional and squamous cell carcinomas are found (53). Hyperplasia induced by FANFT feeding is reversible up to 6 weeks, but after 8 weeks the changes are irreversible and will result in formation of carcinomas in all animals (57). Furthermore, a dose-dependent relation between FANFT-dose and tumor formation could be demonstrated (58). Some FANFT-induced tumors are transplantable in syngeneic rats by orthotopic transplantation, i.e. injection of single-cell suspension in bladder wall of recipient rats (59). Biochemical analyses of FANFT induced tumors revealed an increased expression of ras and myc oncogenes (45,60).

Soloway et al. established five cell lines from FANFT-induced tumors arisen in the urinary bladders of adult C3H/He mice. All cell lines are transplantable into syngeneic mice. One of these cell lines, the MBT-2, is widely used for immunotherapeutical and chemotherapeutical studies (62). The MBT-2 tumor is growing s.c., but more importantly also intravesically (63,64). From the original tumor different metastatic sublines have been derived with an implantation site-dependent difference in metastatic ability (65).

Some other chemicals, such as N-2-fluorenylacetamide (FAA), DMBA, methylcholantrene (MCA), and Bracken Fern (Pteris aquilina) are able to induce bladder tumors (34,66-68). After 4 months of FAA feeding, hyperplasia occurred and after 6-8 months squamous and transitional cell carcinoma appeared. Three cell lines of the squamous cell carcinoma have been established (69). Normal bladder epithelium of C57BL/Icr-a^f mice undergoes neoplastic formation in vitro after a brief exposure to DMBA (68). From these cells, cell lines have been derived, which can be transplanted in bladders of syngeneic mice (70). Implantation of MCA pellets in bladders of F344 rats can induce tumor formation. Three of these induced tumors have been serially transplanted (67).

Oyasu et al. developed the "heterotopically transplanted rat urinary bladder" model (HTB) (71-73). This model consists of an urinary bladder from male F344 rats transplanted into the gluteal muscles of syngeneic recipients with an attached s.c. placed reservoir. HTBs which were exposed to a low dose of MNU developed only low-grade, low-stage carcinomas, whereas bladders exposed to a higher dose of MNU or another carcinogen frequently developed high-grade, high-stage carcinomas (72). Normal urine may contain tumor promoting substances, since HTBs receiving

normal urine had a higher incidence of carcinomas than those receiving normal saline (71).

Most induced tumors consist of both poorly differentiated transitional cell carcinoma and squamous cell carcinoma. In human bladder cancer in the western world, however, pure squamous cell carcinoma is relatively infrequent (3-6% of all bladder cancers) (74). Also from a clinical perspective squamous cell carcinoma differs significantly from transitional cell carcinoma. At time of initial presentation, 86-100% of squamous cell carcinomas demonstrate invasion of the bladder wall and 5-year survival rate is low (7-20%) (75).

1.2 Virus-induced tumors

Polyoma virus, simian virus (SV40), and adeno virus 7 have been shown to produce renal tumors in hamsters and other rodents (6,76). A herpes virus induces renal tumors in the leopard frog (Rana Pipiens). These tumors are generally multicentric, bilateral and metastasize to lung, liver and other viscera. This so called Lucke tumor provides an opportunity to study the natural history of a prevalent, naturally occurring, virally induced neoplasm (76). Claude found spontaneous renal adenocarcinoma in 40% to 60% of BALB/c mice. These tumors are serially transplantable; unfortunately, intratumoral virus-like particles were identified (77). From this tumor two other models were developed, the MKT-CD1 and the RAG model (78).

1.3 Spontaneous tumors

Spontaneous tumors are tumors that arise in animals which have not been deliberately exposed to any carcinogenic agent and which are of strains not known to harbor any vertically or horizontally transmitted oncogenic virus (5).

1.3.1 Renal tumors

Spontaneously occurring renal tumors are rare in laboratory animals, the incidence ranges from 0.0004% to 0.2% (13). Fortner found one spontaneous kidney tumor among 246 Syrian hamsters (79). In two studies no kidney tumors occurred during the life-span of 86 and 352 F344 rats, respectively (80,81). Thompson et al. reported 1 kidney tumor, a nephroblastoma, in 125 Sprague-Dawley rats (82). In a study with the aim to find spontaneous prostatic tumors, 4 renal cancers were found in 300 normally aged ACI rats (chapter VII, this thesis). In contrast to these low tumor occurrences, Eker and Mossige reported a line of Wistar rats in which 37 out of 89 rats (41.6%) developed a renal adenocarcinoma (83). They propose the involvement of a dominant genetic element in the etiology of these lesions. Spontaneous murine tumors have been reported also, e.g. Vandendris described the

RC-tumor, a spontaneously arisen low-immunogenic tumor in CDF1 mice, which microscopically appears as an anaplastic tumor (84). In a review, Murphy reports on the occurrence of spontaneous primary renal tumor in domestic animals, rabbits, cattle and horses and other wild animals like birds and snakes (12).

The two best characterized spontaneous kidney tumors are the Renca adenocarcinoma of Balb/C mice (85) and the rat renal cell tumor in the Wistar-Lewis rat (3). The Renca tumor is an anaplastic tumor of renal origin, it is transplantable by different routes, intramuscularly, intraperitoneally, intravenously, and subcutaneously, but most effectively intrarenally. The tumor metastasizes from an intrarenal implant to the regional lymph nodes, lungs, liver, and spleen, as well as to some other organs. The immunogenicity is relatively low. Tumor-bearing mice routinely die between 35 and 45 days; tumor doubling time is approximately 7 days. The rat renal cell tumor arose spontaneously in the kidney of a male Wistar-Lewis rat and has been maintained by serial transplantation (chapters II and IV, this thesis). Further characterization revealed a histological appearance of a clear cell renal cell carcinoma, with ultrastructural presence of desmosomes (86). Intermediate filament expression pattern studies showed, like most human renal cell tumors, coexpression of vimentin and cytokeratins (86).

1.3.2. Prostatic tumors

Spontaneous prostatic carcinomas are rare in non-human mammals. Rivenson and Silverman, in their bibliographic survey of reports of prostatic carcinomas among laboratory animals published between 1900 and 1977, found no reports of cases in mice (87). In 1983, Waymouth et al. described two spontaneous prostatic tumors in old (>700 days) mice (88). In a group of 55 untreated virgin female Mastomys (Rattus Natalensis) 1 prostatic adenocarcinoma and 4 proliferative hyperplasias were described (89). Furthermore, spontaneous tumors in hamsters and a monkey were found (4,34,79). Leav and Ling studied twenty cases of prostatic cancer in dogs and found clinical, morphological and pathogenetic similarities with the disease in man (90). The cancers, for example, occurred among dogs of 6 years and older and bone metastases were not rare.

In a histological study of grossly normal prostates of 1775 F344 rats, 64 adenomas and 7 adenocarcinomas were found (3.6% and 0.4% respectively) (91). The three best characterized models of prostatic adenocarcinoma among laboratory rats are the Pollard tumors, the tumors of the ACI/seg rat and the Dunning tumors. Spontaneous, metastasizing, prostatic adenocarcinomas develop in 10% of germ free Wistar rats, at 32-40 months of age (92). Further characterization of the Pollard tumors have yielded four cell lines (PA I, PA II, PA III and PA IV). All cell lines can be cultivated in vitro with retention of tumorigenic and metastatic capacities (93-96).

In 1975, Shain and coworkers identified spontaneous adenocarcinomas of

the ventral prostate in 7 of 41 aged (34 to 37 months old) ACI rats (97). To confirm these data and to study the pathology of the disease, Ward et al. examined the prostates of 201 ACI rats, at 24 to 40 months of age (98). They found an age-related increase in the incidence of prostatic tumors. At the age of 33 months 95 to 100% of the rats have intraalveolar prostatic atypical hyperplasias, and 35 to 40% exhibit invasive carcinomas. In vitro cell lines have been established from an ACI prostatic tumor (99,100). However, the tumor from which the cell lines were derived was obtained after 4 months treatment with testosterone containing silastic capsules. Surprisingly, we did not find prostatic tumors in a cohort of 300 normally aged ACI-rats, as described in chapter VII of this thesis.

In untreated Copenhagen rats the incidence of microscopic prostate cancer is 10%, the incidence of grossly manifest prostatic cancer is <1% even at 36 months of age (101). In 1961, W.F. Dunning discovered a prostatic tumor in a 22 months old inbred Copenhagen rat (102). Following serial in vivo passaging of the original R3327 tumor, different sublines were obtained. Isaacs and Hukku described the complete genealogy of all these R3327 sublines, which widely differ in their histology, growth rate, androgen sensitivity and metastatic abilities (103). From most in vivo lines, in vitro cell lines have been established, that retain their phenotypic characteristics (104). All lines are transplantable in Copenhagen rats and the F1 generation of Fisher x Copenhagen rats.

1.3.3 Bladder tumors

Few spontaneous bladder tumor models are available up to now. The Brown Norway rat has a high incidence of spontaneously occurring urothelial tumors of the bladder and ureter, 35% of the male and 3% of the female rats developed bladder tumors, which are transplantable in syngeneic rats (105,106). The invasive tumors consisted of transitional epithelium, but also areas of squamous metaplasia were observed, squamous cell carcinoma, however, was not common. In the Nb rat a single transplantable transitional cell tumor has been described (107). In DA/Han rats 54% of male and 14.4% of female rats develop spontaneous urinary bladder tumors, the incidence increases with age of the rats (108). The most frequent tumors found in the DA/Han rats were solid type transitional cell carcinomas; 0.3% of the male and 9.6% of the female rats had SCC (108).

1.4 Xenografts in nude mice

In vivo models using human tumors offer an important tool in the study of tumor biology, such as patterns of growth and differentiation and responses to treatment. However, heterotransplantation of human tumors generally results in rejection of the graft because of histoincompatibility between donor and host. Exception of this occurs in cases in which the animal host has been rendered

immunologically unresponsive, as by thymectomy, sublethal irradiation or use of antisera against recipient lymphocytes, or when tumors are transplanted in immunologically privileged sites, such as the hamster cheek pouch (109).

In 1962, Isaacson and Cattanach first described a hairless mouse mutant (110,111). Flanagan studied the genetics of the mutation and confirmed that the lack of hair was determined by a recessive autosomal gene, for which the mutant mice were homozygous (112). Pantelouris demonstrated the absence of a thymus and consequently an impairment of cell-mediated immune functions (113). However, humoral antibody formation (B-cell mediated) is only slightly impaired, whereas the activity of other T-cell-independent defense mechanisms, such as NK cells and macrophages are even increased in comparison to normal mice of the same strain (114-116).

Following the demonstration of Rygaard and Povlsen in 1969 that human tumors grow in athymic mice, a large number of serially transplantable xenografts of different human tumors have been established (117). The implantation site of the primary tumor appeared to be of critically importance (118). The xenograft model has the advantage of providing large quantities of tumor tissue in a reproducible manner and a 'physiological' milieu as compared to tissue culture (35). The limitations of this model, however, include differences between host and human metabolism, risks of contamination by transformed host cells or viruses, risks of cross-species immunological interactions, low metastatic range and of course the abnormal host immune functions (35). The xenograft model has shown to have limited value in the selection of the optimal chemotherapy for the individual patient, due to the low take rate and slow tumor growth (119). However, drug tests against a panel of human tumor lines can predict clinical effectivity of these drugs (120).

1.4.1. Renal tumors

A great number of renal xenografts has been described (2,121-130). Clayman et al. could successfully transplant 7 out of 29 primary human renal cell carcinomas into nude mice. It appeared that only tumors with a nuclear grade of 3 or 4 could grow after implantation (129). The authors, therefore, suggest that the nude mice may act as an effective filter to distinguish between aggressive and non-aggressive high grade renal cancers and that tumor take is indicative of impending metastatic disease and death. Similarly, Ebert et al. established 63 in vitro cell lines out of 498 successive attempts. They found that these cell lines, which were able to survive 10 passages and 1 year in continuous culture, were derived from tumors which exhibited clinically aggressive features, e.g. metastases or main renal vein invasion (2). Conversely, only 14.2% of the metastatic tumors could be established in vitro, which implies that biological aggressiveness is necessary but not always sufficient for establishment of a cell line. Twenty-six of 33 (79%) cell lines formed tumors in nude mice (2). In contrast to the take rates of Ebert and Clayman,

Knöfel et al. showed a hundred percent take rate of 30 human renal cell carcinomas implanted into nude mice (130). The "bioassay" indicated by Clayman, therefore, appears not useful in general. The discrepancy between these studies could be secondary to the use of different mouse strains (NIH Swiss versus NMRI) or to differences in the age of the mice at time of implantation (4 to 6 weeks versus 6 to 8 weeks) (130).

Naito et al. established RCC tumor lines with different phenotypic characteristics, including differences in invasion and metastasis in nude mice (131). The various sublines have been isolated from a single surgical specimen by different procedures. Hashimura et al. also described two cell lines growing in vitro and in nude mice which were established from the same patient, either from the primary tumor or from the renal vein effluent of the perfused tumorous kidney (132).

1.4.2. Prostatic tumors

Due to the low take rate after heterotransplantation in nude mice, the number of prostatic cell lines and xenografts is limited. For example, van Steenbrugge et al. established 3 permanently growing tumor lines out of 150 transplantation attempts (133). Likewise, Reid showed that none of 63 surgical specimens of prostatic adenocarcinomas transplanted into 145 nude mice developed into a growing neoplasm (134). However, when mice were treated with anti-mouse-interferon serum, implantation of two primary prostatic cancers gave rise to transplantable tumor lines. This effect has been advocated to the complete blocking of the augmentation of NK cell activity in the nude mice (134). Wagner et al. could increase the take rate of prostatic cancer and BPH specimens by implanting dihydrotestosterone containing silastic implants in the nude mice (135).

Upto now 16 different prostatic xenografts have been described (Table I). Eight lines originated from primary tumors, whereas the remaining lines are derived from metastatic lesions. In vitro cell lines have been established from half of the xenografts. Seven xenografts grow androgen dependently (LNCaP, PC82, HONDA, PC-EW, TEN/12, PC-EG, JCA-1), which indicates that these lines have a preferential growth in intact male nude mice, as compared to female or castrated nude mice (133,136-149).

The original EB33 line grows hormone independently (136). However, after in vitro cloning, 23 out of 111 clonal cell lines revealed a suppression of growth of more than 50% in androgen-free media (150,151). The androgen-dependent clones can be stimulated in vivo with DHT to significantly faster growth.

The DU145 tumor line is derived from a brain metastasis (137,152). After heterotransplantation in nude mice no metastases were found. Kozlowski et al. report that implantation of silastic tubes of beta-estradiol into nude mice prior to tumor implantation increases the incidence of spontaneous metastasis from 0 to 3 out of 10 mice (153).

TABLE I. Prostatic tumor xenografts

Cell line[a]	Origin	Growth capacity in vitro	Growth capacity in vivo[b]	hormone-dependency[c]
EB33 (136)	primary tumor	+	+	-
DU145 (137)	metastasis to brain	+	+	-
PC3 (138)	metastasis to bone	+	+	-
LNCaP (139)	metastasis to lymph node	+	+	+
PC82 (140)	primary tumor	-	+	+
PC93 (141)	primary tumor	+	+	-
HONDA (142)	metastasis to testis	-	+	+
PC-EW(143)	metastasis to lymph node	-	+	+
9479 (144)	metastasis to bone	-	+	-
TEN/12 (145)	primary tumor	-	+	+
TSU-Pr1 (146)	metastasis to lymph node	+	+	-
PC133 (133)	metastasis to bone	-	+	-
PC135 (133)	primary tumor	-	+	-
PC-EG (147)	primary tumor	-	+	+
PPC-1 (148)	primary tumor	+	+	-
JCA-1 (149)	primary tumor	+	+	+

a references, in brackets
b implantation in nude mice
c androgen dependent means a preferential growth in intact male nude mice, as compared to female or castrated nude mice

In 1979, Kaighn et al. characterized the PC3 line, which is derived from a metastatic bone lesion (138). S.c. injection of PC3 cells results in spontaneous metastases to the draining lymph nodes and lungs in more than 50% and 13% of mice, respectively (154-156). Metastases to other organs, including the skeleton, were not observed (156). Different metastatic sublines have been isolated (155). The 1-LN-PC-3-1A (1-LN) subline was derived from a spontaneous lymph node metastasis. The 1-LN spontaneously metastasizes to both regional and distant lymph nodes, and pulmonary micrometastases are observed in 30-50% of mice from which the primary tumor is excised (155,157). Kozlowski et al. also described aggressive in vivo derived variant cell lines (153,158). Investigations comparing the aggressive variants with the parental line showed a significantly greater plasminogen activator activity in the aggressive variants (159).

The LNCaP cell line is developed by Horoszewicz from a metastatic lesion and is androgen-dependently growing in vitro (139). By using the technique of limiting dilution different sublines have been developed (160). Cytogenetic comparison of androgen responsive and unresponsive sublines showed preservation of the original parental karyotype and additional, subline specific, marker chromosomes (161). In vivo propagation of the tumor is possible in 62% of intact male nude mice and in 22% of castrated mice (139). In our own experience 2 out of 10 mice developed tumors after s.c. injection of 4 x 10^6 cells of the LNCaP.FGC subline. Transplantation of these tumors resulted in a tumor take in 15 of 40 mice.

1.5.3. Bladder tumors

After heterotransplantation of human bladder cancer tissue into immune-deprived and nude mice some short term and serially transplantable xenograft lines have been established (35). Sufrin et al. implanted 9 low-grade and 11 high-grade transitional cell carcinomas in a total of 104 nude mice (162). Successful takes were observed in 40% of the tumors. The stage of the tumor, histological grade, multicentricity, recurrence rate and clinical prognosis were not related to the success or failure of graft takes in the nude host (162). Naito et al. confirmed these data, they implanted 30 transitional cell carcinomas and one squamous cell carcinoma in nude mice and obtained s.c. growth in 8 of 31 tumors (163). Serially transplantation was only possible with the squamous cell carcinoma. Furthermore, Matthews et al. showed an 39% successful xenograft growth (7/18), four of these tumors have been passaged successfully (164). Huland could transplant and passage 5 out of 16 bladder cancers (165). Moreover, Naito et al. successfully transplanted 3 out of 8 transitional cell tumors of the upper urinary tract (166).

In immune deprived mice Kovnat could generate progressively growing xenografts from 20 out of 53 tumors (38%) (167). Also in these studies no evident correlation between success of xenografting and stage, grade, or clinical course of the disease was found.

Russell et al. found growth of primary implants in 11 of 20 specimens, from these tumors serially transplantable xenografts have been established (168,169). Each xenograft reflects the histological and ultrastructural features of its tumor of origin. Substantial heterogeneity, however, is present within the morphologically similar tumors. Studies with these lines may help to explain the diverse natural history of bladder cancer.

A great number of immortal cell lines for bladder cancer have been described (170,171). Some of these lines are tumorigenic in nude mice after s.c. inoculation. Two different sublines of the T24 cell line, originally described by Bubenik (172), differed highly in their tumorigenicity (173). One subline was non-tumorigenic, whereas the other subline produced tumors in 100% of the mice. The latter line contained fewer chromosomes and the authors suggest that tumorigenicity may be related to the loss of a regulatory gene (tumor-suppressor gene?) (173). We have also injected T24 cells, obtained from two different sources, in nude mice. Seven months after s.c. injection of 10^7 cells in 1/10 or 2/10 mice tumors developed. Further transplantation of these tumors was successful in all mice (18/18 and 20/20 respectively).

Bladder tumor cell lines have been successfully transplanted in the urinary bladder of nude mice. Comparison of tumors from the invasive EJ and the non-invasive RT4 cell line revealed that the invasive cells contained an endogenous mutated H-ras oncogene (174). Furthermore, the invasive cells have an enhanced Cathepsin B production (175). Cathepsin B is a proteinase with the ability to degrade laminin, a basement membrane component, and thereby facilitating the invasion of tumor cells into the surrounding tissues. Transfection of normal or mutated ras-oncogene into RT4 cell converted these cells into an invasive phenotype (176). Therefore overexpression of normal or mutated ras genes can be implicated in bladder tumor progression.

1.5 Immunogenicity of animal tumors

Human tumors fall, with rare exceptions, into the category 'spontaneous' as defined here (5). Syngeneically transplanted tumors of strictly spontaneous origin usually are non-immunogenic, and are therefore, according to Hewitt, by definition the only appropriate models for clinical cancers (5,177). The current consensus is that tumor specific antigens do not exist, and that all "neoantigens" are shared to some extent with normal tissues (178). In 1953, Foley et al. tested the antigenicity of spontaneous mammary tumors and obtained a negative result, whereas methylcholantrene induced tumors were immunogenic (179,180). Hewitt et al. also found no induction of immunity against 27 spontaneous murine tumors (181). Middle and Embleton obtained similar results in 28 naturally arisen tumors (182). In a previous paper, however, they found that a proportion of spontaneous tumors was

clearly immunogenic (183). Virus-induced tumors express common virus-coded antigens and are usually strongly immunogenic (177). Chemically induced tumors also tend to express individually distinct antigens. Depending upon the carcinogen the degree of immunogenicity is variable, but a large proportion of chemically induced tumors are immunogenic (177). Spontaneous arisen tumors, therefore, seem to provide the best tumor model for immunotherapeutical studies (5,184). Care must be taken for the possibility of incompatibility reactions due to genetic deviations between host and tumor in the course of time (185). Freezing of tumors and returning to the original tumors after a certain number of transplant generations can prevent this genetic drift (186).

2 CYTOKINES USED IN THIS STUDY

2.1 Interferon

Interferon (IFN) was discovered in 1957, when Isaacs and Lindenmann observed the phenomenon of viral interference (187). Viruses could trigger IFN-secretion by infected cells, whereas IFN rendered cells resistant to infection caused by the offending virus as well as from a variety of other viruses. In 1980 a committee devised a system for the nomenclature of IFN and accepted a definition for IFN: "to qualify as an interferon, a factor must be a protein which exerts virus nonspecific, antiviral activity at least in homologous cells through cellular metabolic processes involving synthesis of both RNA and protein" (188).

Up to now three major classes of IFN are defined on basis of chemical, antigenic and biologic differences: alpha-, beta-, and gamma-IFN. These three classes of IFN seem to be present in all mammalian species (189,190).

Natural IFN-alpha ("leukocyte-IFN" or "lymphoblastoid interferon") is produced by leukocytes and macrophages (191). Multiple genes code for two families of more than 20 structurally related IFN-alpha proteins (192-194). In general, human alpha-IFNs are polypeptides of 165-166 amino acids, with at least 70% sequence homology between individual molecules (189,195). The human IFN-alpha genes are all clustered on chromosome 9 (196,197). Natural IFN products obtained from white blood cells of buffy coats are indicated by the symbol (Le), while the designation (Ly) is reserved for IFN-alpha derived from cultured lymphoblastoid cells. Recombinant DNA technology has led to cloning and expression of individual IFN genes in bacteria and eukaryotic cells (198,199). Three human recombinant IFN-alpha preparations are currently in use, IFN-alpha2a, -2b, and -2c.

Natural IFN-beta ("fibroblast interferon") is produced by fibroblasts, and is encoded by a single gene, which is structurally similar to the IFN-alpha genes and also located on chromosome 9 (189,196). There is 29% homology between IFN-alpha and -beta amino acid sequences (200). Upon induction of IFN with poly(I:C) in diploid fibroblasts another human IFN, IFN-beta2, has been reported (189). This IFN appeared to be structurally distinct from the major form of IFN-beta or other known IFN species and has been renamed to interleukin 6 (IL-6) (190).

Natural IFN-gamma ("immune interferon") is secreted by activated lymphocytes following exposure to antigens or mitogens (189). NK cells are also powerful producers of IFN-gamma when stimulated with IL-2 or H_2O_2. Human IFN-gamma is a homodimer of 166 amino acids, including a 20 amino acid secretory peptide. IFN-gamma is encoded by a single gene, located on chromosome 12 (201). The isolation, characterization and expression of the genes encoding rat and mouse

IFN-gamma have also been reported. Both cytokines share approximately 40% amino acid sequence homology with human IFN-gamma (202-204). This low homology is probably responsible for the species-specificity.

Next to their antiviral effects, IFNs have the ability to inhibit cell multiplication, i.e. antiproliferative activity. This activity was first described by Paucker et al. in 1962 (205). Two fundamental mechanisms are associated with the antitumor properties of IFN: direct effects on tumor-cells and immunomodulation.

2.1.1 Direct effects

IFN exerts its action through cell surface-specific receptors. IFN-alpha and -beta share a common receptor, as demonstrated by the ability of IFN-beta to compete for the binding of labeled IFN-alpha, and vice versa (189). The gene encoding for the IFN-alpha/beta receptor has been assigned to chromosome 21 (189). Zhang et al. compared IFN activity against two trisomic-21 (T-21), three diploid (D-21) and one monosomic-21 (M-21) fibroblast cell lines, and found that T-21 cells were more sensitive to the antiviral activities of IFN-beta than of IFN-gamma. This difference, however, was not found for the antiproliferative activities of both IFNs (206). The receptor for IFN-gamma has also been cloned and sequenced, and is located on chromosome 6 (207,208). Following IFN binding to cells the complex is rapidly internalized (189).

The intracellular events induced by IFN are largely unknown (209). A new family of proteins, the immunophillins, might be important in the intracellular signal transduction (210). IFNs act intracellularly by transiently inducing or up- and down-regulating genes. IFN-induced proteins include, 2'-5' oligoadenylate synthetase (2'-5' synthetase), P1/eIF 2 alpha protein kinase, Mx protein, indoleamine deoxygenase (IDO), guanylate binding proteins, metallothionein II, thymosin B4, and xanthine oxidase (189,211). 2'-5' synthetase (212,213) and P1-eIF 2 alpha protein kinase are implicated in the antiviral and cell multiplication effects of IFN. In the mouse the antiviral state is controlled by a host gene Mx, unlike the nuclear mouse protein, the human Mx-like protein is localized in the cytoplasm (189,214).

IDO is ubiquitously distributed in normal and malignant tissues. IDO initiates the metabolism of tryptophan, which is an essential amino acid and is necessary for a variety of metabolic processes (215). Addition of tryptophan to tissue culture medium of cell lines blocks the antiproliferative activity of IFN-gamma (216). These observations make it possible to postulate that tryptophan depletion by IDO induction might be one of the mechanisms of action of the in vivo antitumor activity of IFN (215,217,218).

Among the proteins induced by IFNs are the proteins of the Major Histocompatibility Complex (MHC) class I and II proteins (219-227). MHC proteins are essential for the recognition of aberrant cells by the immune system. Class I antigens are cell surface glycoproteins with a polymorphic heavy chain linked to

beta2-microglobulin and are expressed on all nucleated cells (228). Under normal conditions, class II antigen expression is limited to selected cell types, such as B-lymphocytes, macrophages, and activated T-lymphocytes, however virtually every cell type can be induced by IFN-gamma to express MHC class II antigens. Class II antigen expression has also been shown on renal tubular cells and some renal cell carcinomas (229), although the last observation could not be confirmed by others (230). Cytotoxic T lymphocytes kill target cells which express foreign proteins (e.g "tumor" or viral antigen) only if this protein is associated with syngeneic MHC class I antigens. This phenomenon is referred to as MHC restriction, in contrast to the non-MHC restricted cell killing by natural killer cells (231). A decrease in MHC class I expression can contribute to tumor progression and metastasis (232,233). Experiments with transfection of genes encoding class I antigens in MHC-deficient tumors have demonstrated that augmentation of class I expression can reduce tumorigenicity and metastatic potential and increase immunogenicity (233,234). T-helper cells recognize antigen in association with MHC class II molecules after this antigen has been processed in antigen-presenting cells, such as macrophages, monocytes and dendritic cells (226). T helper cells promote the proliferation and maturation of antibody-producing B-cells. Class I antigens are induced by all three types of IFN, but only IFN-gamma induces class II. Induction or increase of MHC expression may increase the hosts ability of immune-mediated tumor destruction.

In addition to the MHC molecules, antigen-independent interactions between adhesion molecules are required for initiation of a specific immune response. Lymphocyte function associated antigen-1 (LFA-1), a member of the integrin family found on most leukocytes, participates in cell-cell interactions during T lymphocyte help, during killing by both cytotoxic T lymphocytes and NK cells, and in adhesion of leukocytes to endothelium (235). Intercellular adhesion molecule-1 (ICAM-1) is a member of the immunoglobulin superfamily (236), and is expressed not only on hematopoietic cells, but also on endothelial cells, fibroblasts and malignant cells (237,238). ICAM-1 is a counter-receptor for LFA-1; its importance in T cell functioning has been demonstrated by blocking T-cell killing with antibody to ICAM-1 (236,239,240). IFN-gamma and TNF cause strong induction of ICAM-1 on a wide variety of tissues including renal cell carcinoma, and greatly increase binding of lymphocytes and monocytes through their cell surface LFA-1 (236,240-242). This mechanism might be favorable for an increased anti-tumor host response.

Furthermore, IFNs are associated with other cell differentiating effects (244). The synthesis of hormone receptors is considered a function of more differentiated tumors. Alpha-IFN treatment increased the expression of estrogen-receptors on two human breast tumor cell lines, and sensitized these to the antiproliferative effects of the anti-estrogen Tamoxifen (245). Likewise, IFN-beta increased estrogen and progesterone receptors on another human breast cancer cell line (246). Glucocorticoid receptors are induced on a human myelogenous cell line

(247) by IFN-alpha and -beta, and IFN-beta enhanced the production of estrogen and progesterone receptors on human endometrial cancer (248). Induction of androgen receptors on prostate cancers cells could also make these cells more susceptible for hormonal therapy. Immunocytochemical analyses revealed relatively high levels of androgen receptors in the androgen-responsive Dunning R3327H-tumor (249). The androgen receptor mRNA level was about 75% compared to the normal rat dorsal prostate (100%). Androgen independently growing tumors, like the Dunning R3327MatLyLu, had very low levels of androgen receptor mRNA (<5%). In the experiments presented in chapter V of this thesis, we treated rats carrying the H-tumor with rat-gamma-IFN and/or TNF. Castration of these rats, after cessation of cytokine therapy, did not produce a greater tumor regression in comparison to the control group, indicating that in this tumor the cytokines did not induce androgen receptors.

IFN treatment of cells can also lead to decreased expression of genes. Gamma-IFN inhibits the expression of c-erbB-2 gene in ovarian carcinoma cell lines (250). IFN modulates the expression of several other oncogenes, such as c-myc, c-fos, c-Ha-ras, v- or c-src (189). Recently it has been shown that IFN-alpha/beta can selectively down-regulate the expression of mitochondrial genes in both human and murine cultured cell lines, which is a new pathway for IFN-mediated responses (251).

2.1.2 Indirect, immunomodulatory effects of IFN

IFNs efficiently enhance the cytotoxic activity of natural killer (NK) cells. This effect can be demonstrated and quantitated by preincubating lymphocytes in the presence of IFN and then testing their cytotoxic ability against target cells unable to induce IFN production (231). All three types of IFN are able to induce NK cell activity, IFN-gamma, however, enhances NK cytotoxicity to a lower extent and with slower kinetics than IFN-alpha or IFN-beta (231). Although almost all IFN-alpha species induce NK cells, IFN-alphaJ, with potent antiviral and antiproliferative activities, fails to do so. IFN-alphaJ can block the NK stimulatory activity of other IFN-alpha species, and compete with these IFNs for attachment to the IFN-receptor on NK cells (252). In this comparison of multiple alpha-IFNs which differed in only a few amino acids, it has been suggested that the tertiary structure of the protein regulates the ability to stimulate NK cells.

Furthermore IFNs, especially IFN-gamma, activate the tumoricidal activities of monocytes and macrophages (253). The IFNs are part of the cytokine network and are able to induce the production of other cytokines, such as TNF-alpha and interleukin-1, by macrophages (254).

Neopterin, a compound derived from guanosine triphosphate (GTP), represents a precursor molecule of biopterin, an essential cofactor in neurotransmitter synthesis. In monocytes and macrophages neopterin-release is

activated by IFN (255,256). By measuring parameters that are induced by IFN treatment, such as neopterin and ß2-microglobulin, it is possible to compare activities of different IFNs and to assess the optimal biologically active dose in a clinical setting (257-259). For example, Aulitzky and coworkers showed that a relatively low dose of gamma-IFN, 100 μg (2 x 10^6 IU) once weekly was biologically more active than higher doses (260). The low dose resulted in a nearly maximum rise of neopterin and ß2-microglobulin levels in the serum with minimal side effects, whereby the increments in these markers were predictive for response. Similarly immunological parameters were optimally elevated not at the highest dose level for IFN treatment in patients suffering from melanoma or hairy cell leukemia (261-263).

2.2 Tumor Necrosis Factor

The induction of hemorrhagic necrosis by bacterial products has already been described in the early 30's (see for review ref. 264). The substances responsible for these effects are the endotoxins. These are the lipopolysaccharides (LPS) that are found on the outer surface of gram negative bacteria (265). Endotoxin induces "hemorrhagic necrosis" in transplanted tumors, but does not kill tumor cells in culture, which indicates an indirect mechanism of action (265,266).

In 1975, Carswell and Old found that serum of mice treated with endotoxin, 14-21 days after BCG-infection, contained a substance which caused hemorrhagic necrosis of transplanted meth A sarcomas in these mice; this substance was designated tumor necrosis factor (TNF) and is a product of macrophages (266-269). Best effects were found on well-established (7-day) transplants, whereas virtually no effect on 5-day transplants was found. Corynebacteria and Zymosan were as effective as BCG as priming agents for TNF release by endotoxin (266). Human TNF has been purified and characterized by Aggarwal et al. (270) and its gene has been cloned and brought to expression in E. Coli. (271-273).

Other investigators were interested in the mechanism underlying the severe cachectic state accompanying chronic parasitic infections in animals. They observed a marked elevation of plasma very low density lipoproteins (VLDL), which resulted from a clearing defect, caused by a loss of peripheral tissue lipoprotein lipase (LPL) activity (274). Endotoxin treated macrophages and a macrophage cell line produced a monokine that could inhibit LPL activity. This factor was purified to homogeneity by Beutler et al. and named cachectin (274). After cloning of the cDNA for cachectin it was shown that TNF and cachectin are identical (275).

TNF is a protein of 157 amino acids that is synthesized as a 223 amino acid precursor from which a 76 residue signal peptide is cleaved off (271). Mature murine TNF consists of 156 amino acids, the 235 amino acids pre-TNF is 79% homologous to human TNF (276,277). This may be an explanation for the low species specificity of TNF. TNF is encoded by a single gene, located on

chromosome 6 (278-280). The TNF-gene is closely linked to the gene coding for lymphotoxin (LT) or TNF-beta. LT is produced by activated lymphocytes and has anti-neoplastic properties similar to TNF (281,282). About 30% of the amino acids of both molecules are identical. The genes for TNF and LT are located within 8 kB of genomic DNA, between the genes for MHC class I and II antigenes, i.e. located in the region of the so-called Class III MHC genes (280). The close linkage and amino acid sequence homology suggests a common ancestral gene for TNF and LT.

TNF and LT interact with the same membrane receptors. Structural analyses of these receptors by ligand cross-linking and affinity purification revealed a TNF receptor molecule of about 85 kD (283-286). Recently, a molecule of 60 kD was found which represents the biologically relevant TNF receptor, since a monoclonal antibody against this protein uniformly blocked various gene regulatory as well as cytostatic and cytotoxic activities of TNF (287). It is conceivable that p85 may cooperate with p60, e.g. by modulation of ligand binding activity (287). The cell membrane expression of the TNF receptor is enhanced by IFN-alpha (288) or IFN-gamma (284,289,290) and decreased by protein kinase C activating agents (291,292), inhibitors of protein synthesis (293) or drugs that depolymerize microtubules (293). Several mechanisms serve to decrease the number of receptors, such as internalization or reduced synthesis and/or translocation to the plasma membrane (2086). Another mechanism is shedding of receptors, e.g. neutrophils shed TNF receptors to rapidly decrease the number of receptors on their surface (294).

After binding to its receptor, the receptor-TNF complex is internalized and degraded (283). The nature of the intracellular mediators of TNF action have to be identified. Arachidonic acid might play a role in TNF signal transduction, because its release and metabolism is an early event stimulated by TNF (295). Arachidonic acid induces the c-fos oncogene expression in a dose-dependent manner, but did not induce c-jun oncogene expression. Inhibition of the degradation of arachidonic acid by an lipoxygenase inhibitor abolished the induction of c-fos by TNF, but not of c-jun. These data suggest that at least two second messenger pathways are activated by TNF (295). TNF-sensitivity is not solely dependent on the number of functional receptors present on the cell surface or on the ability to internalize TNF, since TNF-resistant cell lines can express equal amounts of receptors as TNF-sensitive cell lines (283,296).

In addition to endotoxin, other compounds are capable of inducing TNF production by monocytes, such as IFN-gamma (297,298), IL-1 (298), IL-2 (299) and TNF itself in an autocrine manner (298,300). Moreover, prostaglandin E2 (301,302), vitamin D3 (303), activated complement protein C5a (304), urinary colony-stimulating factor-1 (CSF-1) (305), granulocyte-macrophage colony-stimulating-factor (GM-CSF) (306) have been shown to induce TNF production. Under certain circumstances hyperthermia can induce TNF synthesis (307). Cellular cyclic AMP and IL-4 act antagonistically on TNF production (299,308).

TNF is predominantly produced by monocytes/macrophages, but several other cell types have also been shown to produce TNF. These include T- and B-lymphocytes and NK-cells, but the quantity of TNF released is minute compared to that produced by macrophages (231,309,310). Recently it has been shown that keratinocytes, after stimulation with endotoxin or UV-light, can release TNF (311). Some tumor cells also have been shown to produce TNF; interestingly some tumor cell lines inherently resistant to TNF express TNF mRNA (312-314). Himeno et al. transfected the TNF-sensitive L-M cell line with a human TNF gene and these cells acquired resistance to TNF (315). Conversely, inhibition of endogenous TNF production by introducing antisense TNF gene into TNF-less sensitive HeLa cells, enhanced TNF sensitivity (315).

2.2.1 Direct antitumor effects of TNF

TNF has potent antitumor effects against tumor cells in vitro (316-319). However, TNF is not equally cytotoxic to all tumor cells and some tumor cells are even resistant (316,319,320). Evenmore, the antitumor activity is dependent on the in vitro test used (321). In vitro antitumor activity of TNF can be enhanced in combination with chemotherapeutic agents directed against DNA topoisomerase II (322-326). In contrast, TNF augmented the growth of diploid fibroblasts (316,327), and has potent fibroblast chemotactic activity (328).

Cell death can occur by two distinct mechanisms, necrosis and apoptosis. Necrosis is the classical form of cell death; flocculation of nuclear chromatin together with non-specific cleavage of the cell's DNA is a feature of cells undergoing necrosis (329). During apoptosis, a series of well defined degeneration changes occur within the cell, which result in the degradation of DNA into oligonucleosome chains. This form of cell death is also termed "programmed cell death" (329,330). TNF induces apoptosis in TNF-sensitive cells (331-333).

TNF has been shown to induce hydroxyl radical production in TNF-sensitive cells, resulting in oxidative damage (334,335). Intracellular hydroxyl scavengers, such as glutathione, exert a protective function against this cytocidal effect of TNF (336).

TNF induces expression of the genes for collagenase and the oncogenes fos, jun, and myc (295,337,338). In another study no correlation between TNF sensitivity and ras and myc oncogene expression was observed (339). Furthermore, TNF has been shown to induce EGF-receptors and HLA class I and II expression on tumor cells (340,341).

2.2.2 Indirect, immunomodulatory effects of TNF

TNF augments the activities of cells of the immune system, such as T lymphocytes (342-344), macrophages (344-347) and NK cells (348). In an clinical trial, however, Kist et al. could not confirm the NK cell augmenting activity of TNF (349). Furthermore, TNF has been shown to enhance B cell proliferation and

differentiation (350,351).

Several animal model studies illustrated the immunomodulatory effects of TNF and the need for an intact immune system. TNF causes regression of SA1 sarcomas in normal mice, but not in thymectomized, T-cell deficient, mice (352). Haranaka et al. showed inferior antitumor effects of TNF against meth A sarcoma in nude mice, compared to the antitumor effects in nu/+ mice (353). Furthermore, TNF induces a specific immune response against meth A sarcoma in intact mice (354). Asher et al. observed tumor necrosis in immunogenic and non-immunogenic murine sarcomas, complete regressions, however, were only seen with immunogenic tumors (355). Manda et al. showed no direct, in vitro, cytotoxicity of TNF against several murine tumors, in vivo treatment, however, showed (indirect) antitumor activity (356).

Furthermore, the production of cytokines, such as IL-1 (357), IL-6 (358), GM-CSF (359) and platelet-activating growth factor (360), is enhanced by TNF.

2.2.3 Vascular effects of TNF

Direct effects of TNF on vascular cells in vitro have been reported. These include, in addition to growth inhibiting and cytotoxic effects (361), the induction of cell surface adhesion molecules (LFA-1, ICAM-I) (362), MHC class I antigens (362-364), platelet-activating factor (PAF) (365), monocyte chemoattractant MCP-1/JE (366) and the enhancement of a procoagulant effect on endothelial cells (367). The direct effects favor the accretion of thrombus and the adhesion of neutrophils, monocytes and lymphocytes on the endothelial surface. The induction of fibrin formation also leads to occlusive thrombosis and is associated with an 80% reduction of tumor perfusion (368). In vivo studies confirmed that thrombus formation is an important effect of TNF (369-371). The effects were more extensive in 6 to 15 days old Meth A sarcomas in comparison to 3 days old tumors. Van de Wiel et al. showed that TNF initially induces an active hyperemia that is rapidly converted to a passive hyperemia (372). Furthermore, TNF increases the permeability of endothelial cell monolayers and stimulates formation of inter-endothelial cell spaces in vitro (373). In vivo studies revealed a breakdown of laminin which can cause loss of vascular integrity (372). McIntosh et al. demonstrated that the neovasculature of transplanted and spontaneous tumors in mice is specifically affected by a single dose of TNF (5-20 μg/mouse), but found no effect on the vascularization of normal tissue transplants (374). This difference might be explained by differences in the neovascularization of normal and tumor grafts, but their exact mechanism remains to be elucidated (374).

2.3 Model systems for measuring antiproliferative effects

In vitro experiments enable us to measure the direct antitumor effects of cytokines. In the studies presented in this thesis a modified *double layer soft agar model system* has been used as originally described by Hamburger and Salmon (375-377). In the upper layer a single cell suspension of tumor cells is plated. These cells form colonies which can be counted with an automated colony counter (378). The number of colonies formed during drug treatment is compared to the colony number of the untreated control and is a measure for the antiproliferative activity of that drug. Assessable growth of primary tumors remains a problem due to the low plating efficiencies and clumping artefacts (379,380). To correct for initial cell clumps a cytotoxic control is added, the number of "colonies" counted in these dishes are deducted from the control or treatment groups (381). There is little opportunity to determine the indirect drug activity using the basic clonogenic assay. Inclusion of macrophages in the underlayer could be a possibility to optimize the assay, since addition of macrophages inhibited colony formation more than IFN-gamma treatment alone, due to the secretion of soluble substances by these macrophages, which consist in part of TNF (382). In a small study, Strayer et al. suggest that the soft-agar assay can identify responders for IFN treatment (383). Clinical correlations between in vitro sensitivity and in vivo response to HuIFN-alpha were possible in 7 renal cell carcinoma patients. 4 out of 4 patients with >60% decrease of colony formation responded clinically (2 PR, 2 minor responses), while all three patients showing less than 60% decrease in colony formation had progressive disease (383)

Another in vitro model is the *MTT assay* In this assay the conversion by viable cells of soluble yellow 1-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide into purple colored MTT-formozan is measured (384-386) This assay is usually used for chemosensitivity testing of cell lines. Mickisch et al attempted to adapt the assay to primary tumors by purifying the single suspension by Ficoll density centrifugation (387). However, also in this study the admixture of fibroblasts and measuring growing fibroblasts in stead of tumor cells cannot be excluded. The soft agar assay has a major advantage in this respect, only tumor cells will grow in soft agar and not the fibroblasts

The *multicellular spheroid model* is of intermediate complexity between monolayer cultures in vitro and tumors in vivo. Spheroids are three-dimensional cell aggregates that simulate micrometastases or intervascular microregions of larger tumors (388). Spheroids from cell lines or from primary tumor specimens, show growth kinetics similar to those of tumors in vivo (388,389). The degree of structural or functional differentiation in the primary tumor may be retained in spheroids (388,389). Within a spheroid there is a large spatial variation in cellular proliferation. Generally, most of the proliferating cells are located in the outer three

to five cell layers. With increasing depth into the spheroid cell cycle times prolong and cells become quiescent, i.e. cells arrested in G_0-phase but remaining clonogenic (389). In the central area of spheroids with diameters larger than 300-500 μm there are often large massive necrotic areas. In the studies presented in Chapter III spheroids are cultured with the liquid overlay technique; spheroids are cultured in dishes coated with a agarose gel to prevent attachment to the bottom of the dishes. Spheroids have been widely used in chemotherapy and radiotherapy research (390). Furthermore, spheroids have been used to analyze the interaction between immune cells and solid tumors (391-393).

Treatment of *nude mice* carrying human tumor xenografts give the opportunity to assess the antitumor effects of cytokines *in vivo*. However, both the antiproliferative and immunomodulatory effects of IFN are species-specific. Human IFN-alpha or IFN-gamma have no effect on the mouse immune system as measured by NK cell or macrophage activity and therefore only direct effects of IFNs on human tumor cells can be assessed (394-396).

In vivo testing of tumors implanted in *rats or mice* offer the major advantage that direct as well as indirect, immune-mediated effects, can be measured. The best tumor models for immunotherapeutical studies are spontaneously arisen, non-immunogenic, autochthonous or implanted tumors in syngeneic mice (5,177). Inoculation of in vitro cell lines in rats or mice gives the possibility of comparing in vitro and in vivo effects of drugs. Disadvantages of syngeneic animal models are obviously the use of non-human tumors, and differences in metabolism and pharmacokinetics between rodents and man of the drugs tested.

2.4. Antitumor effects of IFN against urological tumors

2.4.1 Antitumor effects of IFN against renal cell carcinoma

Fleischmann et al. developed a human renal xenograft, JDF-1, which has been maintained in nude mice. IFN-alpha has a dose-dependent growth-inhibiting effect on colony formation in soft-agar and in nude mice of this tumor (397,398). HuIFN-alpha(Ly) had dose-dependent antitumor effects after daily, for 2-4 weeks, s.c. or i.t. administration against the KU-2 and RCC-1 xenografts (399). RecIFN-alpha and -beta showed modest inhibition of cell growth of the ACHN cell line after a 24 hour treatment period. Cell growth was recovered to normal rate and doubling time 24 hours after removal of the IFNs (400). Borden et al. showed dose-dependent antiproliferative effects after 72-120 hours of treatment with HuIFN-alpha and -beta. In these studies HuIFN-beta was more potent then HuIFN-alpha (401). RecIFN-gamma, however, produced a complete growth inhibition of cell growth, and the cell doubling time was increased after removal of this IFN (400). Gohji et al. showed similar effects; recIFN-gamma had a stronger growth-inhibiting effect in comparison to recIFN-alpha and -beta on the KO-RCC-1 cell line

(402,403). The RCC-nu-1 cell line was more resistant, the IFNs only slightly inhibited cell growth. Higher doses of IFN-gamma significantly enhanced the effects of different chemotherapeutic agents (403). In vitro studies against the SN12 cell lines revealed a more effective inhibition of IFN-alpha hybrids against the parental SN12C cell line, whereas recIFN-gamma was more effective against the metastatic subline SN12L1 (404). In contrast, HuIFN-alpha(Ly) or HuIFN-gamma given daily for 17 successive days could not inhibit JRC-11 xenograft growth (405).

Rec.MuIFN-alpha and -beta have significant antiproliferative effects in the MTT assay against the murine Renca tumor (406). RecMuIFN-gamma, however, exhibited only minimal and inconsistent direct growth inhibition. Recombinant hybrid IFN-alphaA/D is the only human IFN with antiproliferative effects against cells of other species, and therefore this IFN showed antiproliferative effects against the Renca cells in vitro. Very low levels of IFN-gamma potentiated the inhibitory effects of IFN-alpha and -beta. In vivo studies showed potent antitumor effects of IFN-alphaA/D alone or in combination with IFN-gamma. In contrast, the combination of IFN-alpha and -gamma only slightly increased survival of nude mice implanted with Renca cells. This implies that long-term survival of mice probably depends on a T-cell mediated response against the Renca tumor. Cured euthymic mice showed immunity against Renca rechallenge (406).

Nanus et al. tested the antitumor effects of IFN-alpha against 16 RCC cell lines and correlated these effects with the expression of six kidney-associated differentiation antigens (407). The expression of one antigen, gp160, correlated with resistance to IFN-alpha, and, conversely, lack of expression of gp160 correlated with sensitivity to IFN-alpha. This correlation was also observed in vivo using nude mice. The authors conclude that the absence of gp160 expression by RCCs may be predictive for sensitivity to IFN-alpha, and, thus, provide a basis for identifying IFN-responsive patients (407).

Although the number of preclinical studies of renal cell carcinoma is rather limited, clinical studies have been performed. The need for new treatment strategies and the promising effects of IFN in tumor models have provided a rationale for these trials. Cumulative results reviewed in several papers show an overall response rate for IFN-alpha of 16% (191,408,409).

Clinical experience with IFN-beta or -gamma is less extensive. The number of patients treated with IFN-beta is too low to draw firm conclusions. Objective response rates are in the range of 10-20% (191,409-411).

A wide range of response rates has been reported in patients treated with IFN-gamma (191,409,411-413). Rinehart et al., for example, reported no responses in 13 patients during a phase I trial (414). In contrast, low dose IFN-gamma has been associated with an overall response rate of 30% (260). However, Goepel et al. have treated 13 patients with the doses used by Aulitzky et al. and found only 8% responders (415).

Different studies have assessed the effects of combinations of IFNs. In three phase II studies 53 patients received either IFN-alpha and -gamma at equal dosages (2 x 10^6 U/m^2) or at equal protein weights (2 x 10^6 U/m^2 IFN-alpha and 0.2 x 10^6 U/m^2 IFN-gamma) or an alternating treatment regimen was used: IFN-gamma 5 x 10^6/m^2, week 1, IFN-alpha 10 x 10^6/m^2, week 2; 2 weeks out of 3. The response rates of these studies are 0/10 (0%), 4/25 (16%) and 1/13 (8%) (416). Foon et al. did not find differences in response rates in randomized three-arm study comparing IFN-alpha alone (2 x 10^6/m^2, three times a week), IFN-gamma alone (1 x 10^6/m^2, three times a week), or the combination of both cytokines (1/21, 1/21 and 2/47 objective responses, respectively) (417). Ernstoff et al., however, found objective responses in 8 out of 30 assessable patients (27%), sequentially treated with IFN-alpha and -gamma (418).

At the departments of Urology and Medical Oncology at the University of Nijmegen two subsequent phase I/II trials were conducted (419,420). An initial group of patients was treated with an escalating dose alpha-IFN and a fixed dose of gamma-IFN. Based on the experience in the first group, a follow-up study was started with a four times higher alpha-IFN dose and the same low gamma-IFN dose. 32 patients were included in the first study with a starting dose of alpha-IFN 6 μg/m^2 = 2 x 10^6 IU/m^2 and a fixed low dose of gamma-IFN (0.1 mg/m^2 = 2 x 10^6/m^2) s.c. two times weekly. After 4 or 8 weeks of treatment, the alpha-IFN dose was escalated with 6 μg/m^2 until the maximum tolerated dose (MTD); which was 30 μg/m^2 (range 6-36 μg/m^2). In 2 patients a CR (7%) and in 6 a PR (21%) was seen resulting in an overall response of 28% (419). In a subsequent study 25 patients were treated with a starting dose alpha-IFN of 24 μg/m^2 and a low dose of gamma-IFN (0.1 mg/m^2); alpha-IFN dose was escalated every two weeks with 6 μg/m^2 until MTD. The overall response rate of this second group was 22% (420).

More comparative studies are needed to establish exactly the role of IFNs in the management of metastatic RCC. All published results so far are gathered from phase I/II studies. Overall response rates, therefore, have to be evaluated and interpreted with great caution. A comparative EORTC phase III trial is ongoing, comparing IFN-alpha monotherapy to alpha- and gamma-IFN combination therapy.

2.4.2 Antitumor effects of IFN against prostatic carcinoma

The androgen-dependent LNCaP cell line is resistant to HuIFN-beta in vitro (139). In contrast, studies using recIFN-alpha and HuIFN-beta showed antiproliferative activity against the androgen-independently growing PC3 and DU145 cell lines, IFN-beta being more effective then IFN -alpha (404,421). HuIFN-alpha also inhibited PC3 cells in a dose-dependent manner, this direct antiproliferative effect is at least partly mediated by cyclic AMP (422).

A limited number of clinical trials have been conducted in prostatic cancer. Complete and partial responses have been reported, but the number of treated

patients is too small to draw firm conclusions about effectivity of IFNs in prostatic cancer (Table II) (423-426). One case report of a complete response has been described in a patient with prostatic cancer receiving 7 - 9 x 10^6 U HuIFN-gamma per day for 12 weeks (427).

Clinical research with cytokines could be hampered by the fact that prostate cancer is primarily and predominantly a disease of metastases to bone. Experience with the use of IFNs in renal cell carcinoma showed that lung metastases are especially susceptible for IFN treatment (428). The primary tumor, bone and central nervous system metastases rarely respond to this type of treatment. However, clinical responses with IFN-alpha and -beta treatment have already been obtained in prostatic cancer patients with osseous metastases (Table III).

Another consideration regarding the clinical applicability of cytokines is the fact that prostate cancer is a very slow-growing tumor. In vivo labelling studies with the thymidine analogue bromodeoxyuridine, BrdU, revealed an extremely low fraction of cells in S-phase (3.5%, range 1.0 - 10.7%) in a series of 46 patients with prostatic cancer (429). Bladder tumors, for example, have a S-phase fraction of 12.3% (range 2.0 - 30.0%) (429). The low mitotic activity could probably be a reason that chemotherapy has had such poor success in prostate cancer therapy. In contrast, for IFN-alpha and -beta it has been shown that resting tumor cells are more sensitive targets for its antiproliferative activity than rapidly multiplying cells in vitro (430). IFNs can inhibit cell proliferation by blocking cells in the G0/G1 phase of the cell cycle.

Table II. Antitumor effects of IFN against prostatic carcinoma, clinical trials

IFN type	administration route	no. patients	regimen	responses	ref.
HuIFN-alpha	?	10	3 or 9 x 10^6 U/day	1 CR, 3 PR	423
HuIFN-alpha	i.m.	14	6 x 10^6 U/m^2 3 days/month	5 CR, 6 PR	424
rIFN-alpha	s.c.	9	5-10 x 10^6 U/m^2 3x/wk	-	425
HuIFN-beta	i.v.	16	6 x 10^6 3x/wk 12 weeks	-	426

2.4.3 Antitumor effects of IFN against transitional cell carcinoma of the bladder

HuIFN-beta produced >20% growth inhibition in 2 out of 3 cell lines after 120 hr. of continuous exposure, whereas HuIFN-alpha was not effective in these cell

lines (401). In a further study the number of cell lines was extended to five and in addition to the naturally produced IFNs, recIFNs were used (431). All cell lines, except the 647V cell line, were sensitive to IFN. No significant difference was seen between natural and recombinant IFN-alpha. Although recIFN-beta was not equivalent in effectiveness to naturally produced IFN-beta, its antiproliferative activity was greater than IFN-alpha (431). In contrast to IFN-alpha and -beta, rec.IFN-gamma significantly inhibited in vitro growth of the 647V cell line (432). RT4 cells were sensitive to the antiproliferative effects of both rec.IFN-alpha and -gamma, combinations of these IFNs gave synergistic effects (433). The growth of the RT4 cell line grown as xenograft in nude mice, was inhibited by HuIFN-beta, when treatment was started at time of tumor inoculation (434).

Table III. Antitumor effects of IFN against TCC, clinical trials

IFN type	administration route	No. patients		regimen	responses		ref.
		TCC[a]	CIS[b]		CR	PR	
HuIFN-alpha	i.m.	3	-	4 x 10^6 U/day, 8 weeks, then 3x/wk	-	3	443
HUIFN-alpha	i.t.	8	-	2 x 10^6 U/day, 3 weeks	6	-	444
HuIFN-alpha	i.m.	8	-	1 x 10^6 U/2 days, 6 months	1	2	445
HuIFN-alpha	intravesical	8	-	50 x 10^6 U/day,	3	3	446
		-	8	8 weeks	-	-	
recIFN-alpha	intravesical	10	-	5 x 10^7 U 2x/wk 6 weeks	-	4	447
recIFN-alpha	intravesical	8	-	54 x 10^6 U/week,	-	2	448
		-	4	8 weeks	1	1	
recIFN-alpha	intravesical	16	-	50-1000 x 10^6 U	4	-	449
		-	19	/week, 8 weeks	6	5	
recIFN-alpha	intravesical	-	38	10^7 U/week or,	2	13	450
		-	47	10 x 10^7 U/week, 12 weeks, then 1x/month	20	9	
recIFN-gamma	intravesical	13	-	1 mg 3x/week 8 weeks	1	-	451

[a] transitional cell carcinoma,
b carcinoma in situ

Grups and Frohmüller evaluated the effects of recIFN-alpha against 17 bladder carcinoma cell lines, and were able to divide these cell lines in sensitive (>50% growth inhibition), semisensitive (25-50% growth inhibition) and resistant (<25% growth inhibition) cell lines (435). The combination of recIFN-alpha, HuIFN-beta and recIFN-gamma effectively inhibited growth of the 639V cell line (435). Encapsulation within multilamellar liposomes augments the antiproliferative effects of recIFN-alpha against the 253J cell line and could overcome the resistance to IFN-alpha of a 253J subline (436). More than 50% of the cells contained intracytoplasmatic liposomes (436).

Differences in IFN sensitivity of different cell lines could not be attributed to the number of IFN receptors per cell or IFN affinity for these receptors (432,437).

Mouse IFN-alpha/beta demonstrated antiproliferative activity in vitro but did not affect MBT-2 tumor growth in vivo (438). Combination treatment with Adriamycin or DFMO did not increase the effect of IFN in vivo (438,439).

Several clinical trials have been conducted with different IFNs (Table III) (440-451). Intravesical instillations were used without any apparent side effects. The studies presented here have shown activity of IFN against superficial bladder cancer as well as against carcinoma in situ.

2.5 Antitumor effects of TNF against urological tumors

The number of studies using TNF as single agent is limited. Heicapell et al. used the tumor lines described by Naito et al. (131,452). They found significant differences among the different cell lines, demonstrating the heterogeneity of cytotoxic effectivity of TNF within a single renal tumor (452). Shibayama found in vitro antitumor activity against the KU-2 cell line (453). Burgers and Donaldson, using different RCC xenografts, both showed in vivo antitumor effects in nude mice, and demonstrated synergistic antiproliferative effects in combination with the chemotherapeutic agent VP16 (454,455).

Shaw et al. treated rats bearing the Dunning R3327 MatLyLu prostatic tumor by intraperitoneal or intratumoral TNF injections (456). Intratumoral administration was most effective in both reducing tumor growth and in prolongation of survival (456). Sherwood et al reported cytotoxicity of TNF against the PC3, DU145 and LNCaP cell lines in vitro (457). Intravenous treatment of nude mice bearing the PC3 tumor resulted in significant tumor growth inhibition (457). Fruehauf et al., however, found that the PC3 cell line was almost completely resistant in vitro to the action of TNF, whereas LNCaP cells were sensitive targets for TNF (458).

Most studies using TNF in TCC have been performed in the murine MBT-2 model. Studies with TNF derived from supernatants of the endotoxin stimulated

J774.1 macrophage cell line showed in vitro cytotoxicity, this crude TNF also significantly suppressed growth of subcutaneous tumors (459). Recombinant TNF alone, or in combination with a low dose Actinomycin D had no cytotoxic effects in vitro, furthermore, intravesical treatment with 10 μg TNF did not reduce the outgrowth of bladder tumors in vivo (460). In contrast to these results, Lee et al. observed a significant reduction in MBT-2 tumor weight with intravesical crude and recombinant TNF treatment in a dose-dependent fashion (461). The tumor incidence was lower with crude TNF, which was not the case with the recombinant TNF. The differences in antitumor activity may be explained by admixture of other cytokines or endotoxins in the crude TNF preparations or by differences between human and murine TNF. In the KK-47 bladder tumor xenograft model, intravenously or intratumorally administered recombinant TNF did not inhibit the growth of subcutaneously implanted tumors (462). Das et al. used the DU-4284 xenograft, implanted under the renal capsule, and found that intravenously administered TNF (50-100 μg/kg 2 or 3 times) significantly inhibited tumor growth (463). In contrast to adriamycin, cisplatin and dactinomycin, VP16 synergistically potentiated the cytotoxic effects of TNF (463).

Several phase I trials with s.c, i.m or i.v. TNF administration have been conducted. The maximum tolerated dose is approximately 150 $\mu g/m^2$ (464-470). Figlin et al. treated 14 RCC and 11 melanoma patients with an escalating dose of TNF (25-75 $\mu g/m^2$/day), daily for 8 weeks and found no objective responses (471). Eisenhauer et al. treated 22 RCC patients with 150 μg TNF/m^2 for 5 days every other week and observed 1 PR and 1 CR (472).

2.6 Combination treatment with IFN and TNF

2.6.1 In vitro studies

Different in vitro and in vivo studies revealed synergistic antiproliferative effects of the combination of IFN and TNF against different tumors. For example, IFN-gamma in combination with TNF has synergistic effects against colon carcinoma (473), cervical carcinoma (474), mastocytoma (475) and lung cancer cell lines (476). Fransen et al. investigated the effects of IFN-gamma and TNF against more than 30 normal and tumor cell lines (477). IFN-gamma and TNF had remarkable synergism against a large number of tumor cell lines. Some cell lines, not sensitive to TNF alone, became highly sensitive when IFN-gamma was present as well. All normal, non-transformed diploid cell lines were insensitive to TNF even in the presence of IFN-gamma (477).

Salmon et al. used the soft-agar assay to test the growth-inhibiting effects of IFN-gamma, TNF and their combination against 102 primary tumors (478). In 68 instances the tumor specimens were tested against IFN, TNF as well as the combination. Exposure to the combination often resulted in a greater antitumor

effect than observed with either agent alone, with at least subadditive effects in 62% of the specimens tested (especially in colorectal and lung cancer). Exposure to the combination reduced the dose of TNF required for significant antitumor activity by about threefold (478). Price et al. studied the cytotoxic effects of IFN-gamma and TNF on primary cultures of leukemia cells, and found additive and synergistic effects in cells from all 17 patients (479). IFN-alpha and -beta also showed synergistic effects in combination with TNF against different cell lines (480-482).

Kavoussi et al. demonstrated synergistic in vitro effects of IFN-gamma and TNF against four different RCC cell lines (483). Pretreatment of tumor cells with IFN enhanced or induced sensitivity of tumor cells to TNF. In vitro studies using five different RCC xenografts were performed in our laboratory (484,485). After original subcutaneous transplantation of small tumor pieces (2 mm^3) in both flanks of Balb-C nu/nu mice, the tumors were passaged every four weeks. Single cell suspensions were used for in vitro tests using a soft agar assay. Drugs were administered as a single application on day 1, colony formation was followed during four weeks in which growth was measured using temporal growth curves (377). A dose-dependent inhibition in colony formation was evident for all five tumor lines in single drug tests. In combination tests 1,500 IU IFN/ml and 3,000 IU TNF/ml were used. All combinations showed synergistic effects, except for the combination of alpha- and gamma-IFN in the NC-65 tumor which had sub-additive effects. The combinations of IFN and TNF showed the best in vitro antiproliferative effects. A heterogeneity towards the direct antiproliferative effects of cytokines in RCC was shown.

Liu et al. performed in vitro studies using the PC3 prostatic cancer cell line and demonstrated antitumor effects of IFN-gamma and TNF monotherapy (486). TNF has synergistic effects in combination with the growth factor inhibitor suramin. The authors, however, did not test the effects of the combination of IFN-gamma and TNF. Up to now, no studies have been performed with the combination of IFN and TNF in prostatic cancer.

Bahnson and Ratliff showed, in an in vitro study, that murine bladder tumor cells (MBT-2), 4 hours pretreated with IFN-gamma were more sensitive to TNF (460). Longer pre-incubation times (24 hours, 48 hours) did not enhance TNF mediated cytotoxic activity. IFN-gamma alone exhibited no antiproliferative activity toward these cells (460).

2.6.2 In vivo studies

IFN-gamma and TNF have synergistic effects in different syngeneic model systems; e.g. for melanoma (487,488), colon cancer (489), methylcholanthrene (MCA) induced sarcoma (490) and breast cancer (491). IFN-alpha induced synergistic effects in combination with TNF against a weakly immunogenic sarcoma model, MCA-106 (492). No improved antitumor effects, however, resulted from

therapy with any cytokine alone or in combination against the non-immunogenic MCA-102 sarcoma (492).

Combination of IFN and TNF also revealed enhancement of their respective activities in nude mice. Human tumor xenografts of breast and bowel tumors (493), ovarian tumors (494) and melanoma (495) were sensitive targets for cytokine combination therapy.

We have performed studies with eight human renal tumor xenografts, which were transplanted subcutaneously (s.c.) into nude mice (496). Of three tested routes of administration, intravenous, intraperitoneal and s.c., only the s.c. route was effective against tumor growth. IFN was given 3 times per week and TNF 3 or 5 times per week in different concentrations. From initial results with the NC-65 tumor 10 treatment regimens were chosen, alpha-IFN 0.5 and 5 ng/gram mouse, gamma-IFN 8 and 80 ng/gram and TNF 500 ng/gram were used and combinations of these cytokines in order to evaluate the effects in the other xenografts. Combinations of both alpha- and gamma-IFN with TNF resulted in a complete growth inhibition of most tumors. All tested RCC xenografts showed different sensitivities towards the cytokines, which may represent the heterogeneity of RCC. The IFN's showed to have optimum doses, which was sometimes a low dose, TNF antitumor effects were dose-dependent. The best antiproliferative effects were found when treatment was started 24 hours after tumor implantation, treatment starting at a tumor volume of 50 mm^3 showed no or slight growth inhibiting effects.

Baisch et al. also showed that the combined treatment of IFN-alpha and TNF was more effective against a RCC xenograft than the single treatments (497).

In contrast to the enhanced antiproliferative effects in vitro against the murine bladder tumor MBT-2, in vivo treatment with IFN-gamma and TNF did not provide additional benefit in comparison to TNF monotherapy (498).

The mechanism of action of the synergism between IFN and TNF remains to be elucidated. Several studies showed that IFN-gamma can induce the number of TNF-receptors on the tumorcell surface (284,289,290). Aggarwall et al., found that IFN-alpha, -beta, and -gamma had synergistic cytotoxic effects in combination with TNF against the ME-180 cervical carcinoma cell line; TNF-receptor-induction, however, occurred only with IFN-gamma treatment and the authors therefore conclude that the induction of TNF-receptors by IFN is not a major mechanism for their synergistic cytotoxic effects (499). Furthermore, IFN-gamma induces the expression of the TNF-gene and the release of TNF (227,500). The DNA-fragmenting activity of TNF is enhanced by IFN-gamma (501). Another mechanism of action could be the induction of IL-6, which was observed during IFN-gamma and TNF combination therapy, but not during treatment with either cytokine alone (502).

2.6.3. Clinical trials

The number of clinical trials performed with IFN and TNF combination therapy is limited. Abbruzzesse et al. performed a phase I trial in patients with advanced gastro-intestinal cancer and observed suggestive antineoplastic activity in biliary and pancreatic cancer (503).

In a phase II study, the efficacy and side effects of TNF 50 $\mu g/m^2$ i.m. and gamma-IFN 0.1 mg/m^2 s.c., 3 times per week, in advanced, progressive RCC was studied (411). 21 patients have been included in this study, 13 males and 8 females. In 14 patients evaluable for response 1 CR and 3 MR have been found, 10 patients had progressive disease. Side effects included high fever, rigors, flu-like symptoms and anorexia in all patients. These preliminary data show a modest activity and a considerable toxicity for the combination of TNF and gamma-IFN in the treatment of RCC.

Otto et al. evaluated in an phase I/II trial the toxicity and efficacy of TNF monotherapy and combination treatment with IFN-alpha2a in RCC (504) Patients received either TNF alone (n=7) with dose escalation from 20 to 300 $\mu g/m^2$ i v, 5 times per week, or IFN-alpha2a alone 12 x 10^6 IU i m. 3 times per week, every week (n=4), or the combination of both regimens (n=5). No objective responses were found in any schedule. Toxicity included fever, chills, flu-like symptoms, nausea, vomiting, malaise, anorexia, hepato- and myelotoxicity The MTD was between 120 and 300 $\mu g/m^2$ for TNF. In a phase II study with the combination of TNF 160 $\mu g/m^2$, 5 times per week, every third week and IFN-alpha2a, 18 x 10^6 IU, 3 times per week every week, 18 patients were included. 14 of these patients are evaluable for response up to now. 2 CR, 4 PR (43% objective responses) and 6 SD were found They conclude that combination treatment of alpha-IFN and TNF is a tolerable treatment for RCC patients. The follow-up in this study is relatively short, and these positive effects need to be confirmed by others.

3. SCOPE OF THE THESIS

In the studies presented in this thesis we have investigated the antitumor effects of IFN and/or TNF against renal, bladder or prostatic tumors in different in vitro and in vivo model systems.

The treatment of choice for localized RCC is surgery. However, nearly 40 to 50% of patients have metastatic disease at time of presentation and will die from their cancer. The median survival of patients with metastatic RCC is approximately 12 months; survival at 2 years is less than 10% (505). As in other types of advanced cancers, therapy of disseminated RCC has been based on the three traditional approaches: surgery, chemotherapy and radiotherapy. None of these have significantly improved patient survival. The observation that spontaneous remissions occur in approximately 0.8% of patients with RCC has led to the concept that the disappearance of distant metastases without therapy may be due to rejection by the immune system (506). This generated interest in the use of immunotherapeutic modalities such as cytokines. IFN is the agent most widely used in the treatment of RCC (section 2.4.1), however, many questions regarding the route of administration, treatment schedules and dosages remain unanswered. The number of (pre)clinical studies using TNF in RCC is limited. Some studies using nude mice showed promising results with IFN/TNF combination treatment (496,497). In **chapter II** we describe the antiproliferative effects of IFN and TNF mono- and combination therapy and the importance of tumor volume at start of therapy on tumor growth of a rat renal cell tumor. Next to cytokine combination therapy, we have tested the growth-inhibiting effects of TNF in combination with radiotherapy on rat renal cell tumor spheroids (**chapter III**). The antitumor effects of the combination of TNF with chemotherapeutical agents directed against DNA-topoisomerase II on in vitro and in vivo growth of this rat renal cell tumor, are described in **chapter IV**.

Hormonal therapy is for almost 50 years, since the pioneering efforts of Huggins and Hodges, the major treatment modality for prostatic cancer (507). Unfortunately, almost all of these patients will relapse to a state unresponsive for further anti-androgenic therapy or chemotherapy. Therefore, new treatment modalities are urgently needed. In **chapter V** we describe the antitumor effects of IFN and TNF mono- and combination therapy against in vitro and in vivo tumor growth of androgen-dependent and -independently growing rat prostatic tumors. Since IFN and TNF combination therapy has not been tested against human tumors we have also performed studies in the nude mouse model system against two human androgen-independently growing xenografts (**chapter VI**).

In chapters II-V we have used spontaneously arisen serially transplantable rat renal- or prostatic tumors. For TCC few spontaneous tumor models are available (section 1.3.3). In **chapter VII** we describe the establishment and characterization of two independently arisen rat bladder tumor (RBT) lines, representing the

progression of bladder cancer from slow growing, non-metastasizing tumors to rapidly growing, metastasizing tumors. This model system may be helpful in the identification of specific markers associated with the progression of bladder cancer.

TCC has already been shown to be effectively treated by non-specific immunotherapeutical agents such as BCG. This treatment, however, has been associated with local and/or systemic side effects. Intravesical treatment with IFN also showed clinical effectivity, but without side effects (section 2.4.3, Table II). The number of experimental studies using TNF is limited (section 2.5). We, therefore, used the RBT model to test the antitumor effects of IFN and TNF mono- or combination therapy against TCC (chapter VIII).

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CHAPTER II

IN VIVO ANTIPROLIFERATIVE EFFECTS OF GAMMA-INTERFERON AND TUMOR NECROSIS FACTOR ALPHA IN A RAT RENAL CELL CARCINOMA MODEL SYSTEM

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ABSTRACT

We have investigated the antiproliferative activities of recombinant rat-gamma-Interferon and recombinant human Tumor Necrosis Factor alpha in a rat renal cell carcinoma model system. The tumor was transplanted subcutaneously, the drugs were administered peritumorally. Gamma-Interferon treatment starting two days after tumor implantation resulted in a dose-dependent growth inhibiting effect. Tumor Necrosis Factor alpha was only effective at the highest concentration. Different combinations of the drugs have additive or synergistic antiproliferative effects. The combination of both highest doses completely inhibited tumor growth without any obvious toxic effects on the rats. Rechallenge of the cured rats with a tumor piece in the contralateral flank did not result in a tumor specific immune response. Shortening of the treatment period to two weeks resulted in an increased lag-period, but finally all tumors started to grow. Furthermore, the anti-tumor effect was dependent on tumor volume at start of therapy. Monotherapy could not inhibit tumor growth of an established tumor. The combination with both highest doses, however, inhibited tumor growth even when treatment was started at a tumor volume of two to five cm^3.

Treatment with gamma-Interferon and Tumor Necrosis Factor is most effective at low tumor burden. These studies suggest that clinical application of these drugs is most effective in an adjuvant setting.

INTRODUCTION

Advanced carcinoma of the kidney is refractory to nearly all forms of systemic therapy. Hormonal therapy can result in a small proportion (0-10%) of objective responses, but these are incomplete and of short duration. Single or combination chemotherapy also gives no clearly definable improvement in patient survival.

Immunotherapy is a relatively new approach in cancer therapy. Biological Response Modifiers (BRMs) like interferons, tumor necrosis factor and interleukins show promising results in experimental model systems and in Phase I and II clinical trials[1,2,3]. The efficacy of interferon (IFN) monotherapy in renal cell carcinoma (RCC) has recently been reviewed[4] and is associated with clinical responses in approximately 15% of patients. Combination therapy with alpha- and gamma-IFN can result in even higher response rates[5]. Lymphocyte activated killer cells combined with interleukin 2[6] and tumor infiltrating lymphocytes[7] also appear to be very promising immunotherapeutic options.

Gamma-IFN is a product of activated T-lymphocytes and has pleiotropic effects[8]. For example, it can directly inhibit the growth of tumor cells in vitro, alter the expression of surface antigens and stimulate NK-cells and macrophages.

Tumor necrosis factor alpha (TNF), originally identified as a substance produced by macrophages exposed to endotoxin, possesses a number of biological activities, particularly anti-tumor activity. TNF causes cytostasis or cytolysis of in vitro cell cultures from a variety of animal and human malignant tumors[9]. However, TNF can stimulate the in vitro growth of normal cells and induce procoagulant activity in endothelial cells and thrombus formation[10], which has been suggested as an indirect tumoricidal effect. In vitro studies on human renal cell xenografts showed that TNF has direct antiproliferative effects on RCC[11]. Furthermore, in vivo studies using murine models, showed that TNF had anti-tumor effects at high doses[12,13].

Combinations of gamma-IFN and TNF can result in the enhancement of their respective anti-tumor activities[12-15] thus providing an even more promising therapeutic approach, that up to now has not been tested for RCC in syngeneic model systems in vivo. Even though phase I and II trials indicate that TNF or gamma-IFN treatment is feasible[2-5], many questions regarding the mode of action (i.e. direct cytotoxic effect vs. indirect immune system mediated effects), the route of administration, treatment schedules and dosages remain hitherto unanswered. Therefore, more basic studies in experimental animals may provide guidelines for the optimal use of BRMs in humans.

In the studies described here, we tested the antiproliferative effects of gamma-IFN and TNF in a syngeneic tumor model system, a spontaneously arisen rat renal cell carcinoma. Histologically and biochemically the tumor closely resembles human RCC[16,17].

Both the direct antiproliferative and immunomodulatory activities of gamma-IFN are species specific. Human gamma-IFN is active in human and not in syngeneic mouse systems and vice versa[18]. Therefore we have used rat-gamma-IFN in our rat renal cell tumor model system, this rat-gamma-IFN showed no antiviral activity with mouse or human cells, both sensitive to their homologous interferon, but rat cells could be protected against both vesicular stomatitis virus and vaccinia virus[19]. In contrast to gamma-IFN, TNF shows little species specificity. Murine and human TNF are 79% homologous at the amino acid level and only the murine natural TNF is a glycoprotein[20]. The in vitro activities of murine and human TNF were compared, and only a very limited degree of species specificity was found[20]. Also in vivo the species preference is not as pronounced as in the case with the interferons[21]. Both murine and human TNF showed synergistic antiproliferative effects with murine gamma-IFN in a mouse model system[13].

The importance of tumor volume at start of therapy was evaluated, and the implications of these findings for clinical use of these BRMs are discussed.

MATERIALS AND METHODS

Animals

Adult male Lewis rats (Charles River Wiga GmbH, Sulzfeld, FRG) were kept 3 rats per cage with a regimen of 12 hours light per day. The animals were allowed access to RMH-TM food (Hope Farms BV., Woerden, The Netherlands) and water ad libitum.

Tumor Line

The rat renal cell tumor line was kindly provided by Dr. R. Babayan, Boston, MA. The tumor was maintained by serial subcutaneous transplantation. For our experiments, the tumor was excised from a tumor-bearing animal, and after removal of normal and necrotic tissue, the tumor tissue was cut into pieces of 50 mg (46-53 mg). The precise weight of each tumor piece was measured on preweighed sterile gauzes on a laboratory scale (Mettler). An 0.5 cm incision was made in the right flank of recipient animals, and after separating the subcutaneous tissue by blunt dissection, the tumor fragment was placed subcutaneously (s.c.). The incision was closed using skin clips (7.5 x 1.75 mm, Aesculap, Germany). In this way a tumor take of 100% was achieved. The tumor did not metastasize from this site.

After complete removal of subcutaneous tumors of 2-3 cm^3 together with overlying skin and subcutaneous fat, no tumors recurred. A direct rechallenge with 50 mg tumor pieces in the contralateral flank did not cause any changes in tumor take or growth rate in 14 untreated rats, suggesting that this tumor is not immunogenic.

Drugs

Recombinant human TNF alpha was kindly provided by Boehringer Ingelheim (Alkmaar, The Netherlands). The specific activity of TNF determined in the presence of Actinomycin D was 6 x 10^7 Units/mg protein as determined in the L-929 cytotoxicity assay. The purity was >99% as determined by SDS polyacrylamide gel electrophoresis and it contained less than 1.0 ng endotoxin per mg protein based on the limulus amoebocyte assay. The original TNF solution was diluted with RPMI 1640 culture medium (Gibco, Paisley, UK) to a final concentration of two, 20, or 200 μg per ml, divided in aliquots and stored at -20^0 C.

Recombinant rat gamma-IFN was produced by a chinese hamster ovary (CHO) cell line transformed with a plasmid carrying the chromosomal gene for rat gamma-IFN and purified as described previously[22]. The cytopathic effect reduction assay was used for estimating gamma-IFN activity. Ratec cells[19] (a continuous rat embryonic cell line) were incubated with gamma-IFN for 18 hours before they were challenged with vesicular stomatitis virus to determine the cytopathic effect. Because no internationally accepted reference standard for rat interferon is available, all titers were determined by comparison with a laboratory standard preparation of gamma-IFN (one mg pure rat gamma-IFN corresponds with 7 x 10^6 Units). After purification by monoclonal antibody affinity chromatography gamma-IFN was freeze-dried for longtime storage. It was reconstituted with Milli-Q to a final concentration of 16,000 or 160,000 Units per ml, divided in aliquots and stored at -80^0 C until use.

Treatments and monitoring

Gamma-IFN and TNF were administered s.c., peritumorally, 3 and 5 times per week respectively, in a 0.5 ml injection volume. Phosphate buffered saline (PBS) was used for the control group. Treatment started two days after tumor implantation or at a tumor volume of 0.2-0.5, one or two to five cm^3 and was continued until the tumors in the control group reached a volume of 10 cm^3. However, in the experiments with shorter treatment periods, two weeks of treatment or a single administration was given. All experiments were carried out in duplicate.

Tumor growth was followed by measurements of tumor size according to the method of Janik et al.[23] by the same measurer throughout each experiment. Three times per week length (l), width (w) and height (h) were measured using a slide caliper. No correction was made for skin thickness. The tumor volume (V) was calculated from the formula

$$V = 0.5236 \times l \times w \times h.$$

Statistical analysis

For each rat, the tumor volume was estimated 6 weeks after tumor implantation (i.e. time when control group reaches volume of 10 cm^3) by log linear regression on the tumor volumes throughout this period. Two-sided t-tests were used

to compare the final volumes of the treatment versus the control groups and to test for additive effects in the combination treatments. $P < 0.05$ is considered as statistically significant. 6 animals were included in each group.

The effect of treatment A is defined as $E_A = V_{control}/V_A$ and the effect of treatment B as $E_B = V_{control}/V_B$, where V_A, V_B, and $V_{control}$ are the estimates for the tumor volumes of treatment A, B or control group respectively. Additivity is defined[24] as $E_{A+B} = E_A \times E_B$ (i.e. $\log E_{A+B} = \log E_A + \log E_B$). A combination of two treatments is synergistic when the effect of the combination (E_{A+B}) is greater than the product of the effects of the separate treatments.

RESULTS

To study the in vivo anti-tumor effects of gamma-IFN and TNF, we used a spontaneously arisen, syngeneic transplantable rat renal cell tumor line[16,17].

Single agent therapy.

Pilot-studies in which we evaluated the influence of administration route on anti-tumor effect showed that i.p. and i.v. injected BRMs were less effective than s.c. injections. Similar observations were made in other rodent model systems[25]. Therefore, we injected gamma-IFN and TNF s.c., peritumorally, in the experiments described.

Gamma-IFN monotherapy (fig. 1A) was started 2 days after tumor implantation. Under these conditions gamma-IFN has an antiproliferative effect whereby the highest dose results in the strongest growth inhibiting effect, suggesting a dose-dependent response (40,000 U gamma-IFN, s.c., 3 times per week gave intermediate effects, data not shown).

When TNF was used as single agent only the highest dose (i.e. 100 μg TNF per rat per day) produces a significant anti-tumor effect (fig. 1B).

In both series of experiments using single agent therapy it appeared that after termination of therapy, the tumor regained its original growth potential (i.e. same volume doubling time, data not shown).

Combination therapy

Reasoning that gamma-IFN and TNF can have additive or synergistic effects, we combined 8,000 or 80,000 U gamma-IFN with 10 or 100 μg TNF (fig. 2). The combination of 8,000 U gamma-IFN and 10 μg TNF had an additive growth inhibiting effect. A tenfold increase of the TNF dose did not improve the anti-tumor effect.

Combinations with the highest dose of gamma-IFN produced the best effects. In the group treated with the combination 80,000 U gamma-IFN and 10 μg TNF, only 2 out of 6 rats developed a tumor (Table I). Moreover, tumor growth

Figure 1

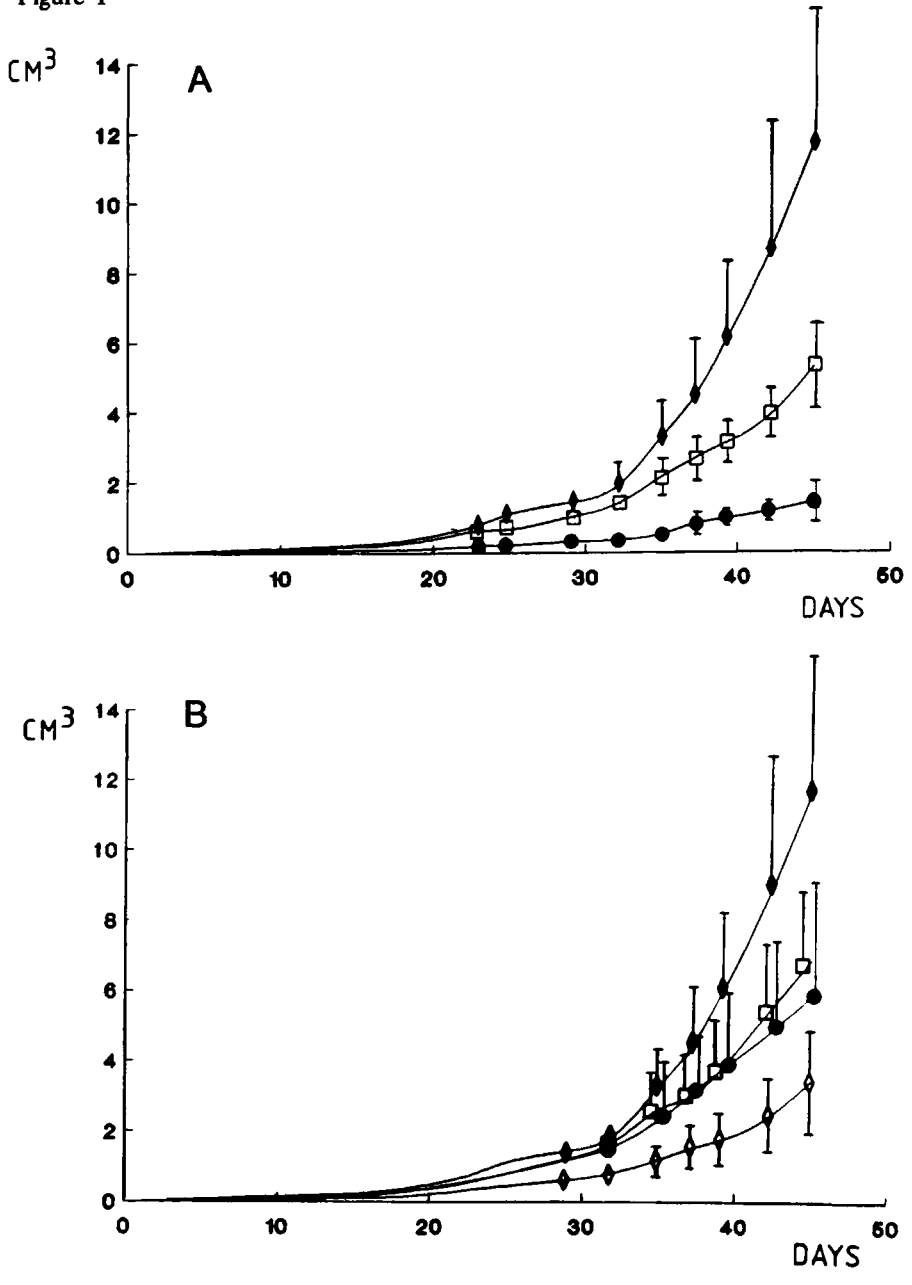


Figure 1. Effect of single agent therapy started 48 hours after tumor implantation.
A: Gamma-IFN monotherapy administered s.c., peritumorally, 3 times per week. ♦—♦ control, □—□ 8,000 U gamma-IFN ($p = 0.05$) and ●—● 80,000 U gamma-IFN ($p < 0.001$).
B: TNF monotherapy, s.c., peritumorally, 5 times per week. ♦—♦ control, □—□ 1 μg TNF (n.s.), ●—● 10 μg TNF (n.s.) and ◇—◇ 100 μg TNF ($p = 0.02$), n.s. = not significant

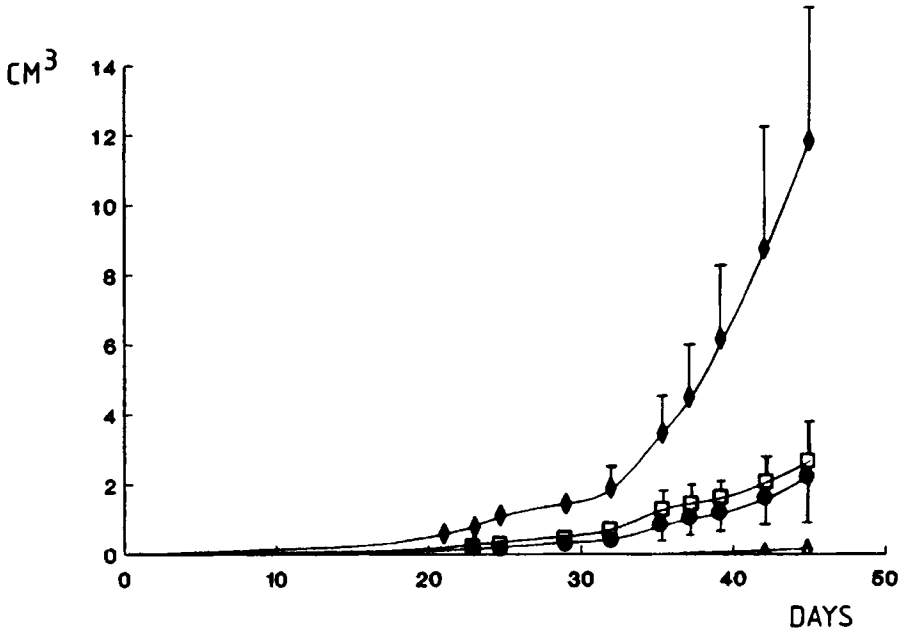


Figure 2. Combination therapy with gamma-IFN and TNF, 3 times and 5 times per week respectively. Treatment started 48 hours after tumor implantation. ◆—◆ control, □—□ 8,000 U gamma-IFN and 10 μg TNF ($p = 0.002$), ●—● 8,000 U gamma-IFN and 100 μg TNF ($p = 0.03$), ◇—◇ 80,000 U gamma-IFN and 10 μg TNF ($p < 0.001$). With the combination 80,000 U gamma-IFN and 100 μg TNF no tumors developed at all.

Table I

Treatment		Bodyweight[c]	Bodyweight	Increase[d]	Cures[f]
IFNa	TNF[b]	day 0	day 42		
0	0	365 (28)	431 (33)	65 (8)	0/6
8,000	0	359 (26)	414 (13)	55 (7)	0/6
80,000	0	373 (20)	ND		0/6
0	1	375 (12)	431 (17)	56 (7)	0/6
0	10	359 (14)	415 (17)	56 (7)	0/6
0	100	356 (14)	415 (25)	59 (15)	0/6
8,000	10	373 (27)	424 (34)	51 (8)	0/6
8,000	100	352 (22)	420 (38)	68 (18)	0/6
80,000	10	364 (21)	422 (24)	57 (12)	4/6
80,000	100	352 (21)	414 (18)	58 (11)	6/6

[a] units rat-gamma-IFN per rat, 3 times per week
[b] micrograms TNF per rat, 5 times per week
[c] weight in grams, standard deviation in parentheses
[d] weight increase in grams, standard deviation in parentheses
[e] not determined
[f] number of rats tumor free for more than 200 days/total number of rats per group

Table I. Effect of s.c. peritumoral administration of recombinant rat-gamma-IFN and recombinant human TNF on bodyweight and number of cured male Lewis rats. Data of a representative experiment.

started 35 days later than the control group (lag-period of 35 days). Significantly, at this dose of 80,000 U gamma-IFN with a tenfold increase of the TNF concentration to 100 μg no tumors developed at all. Both combinations have synergistic effects ($p < 0.001$). Because 200 days after tumor implantation no tumors were found, these rats were considered cured.

Effect of duration of therapy

Having found a drug combination that could prevent tumor formation completely, we sought to gain more insight regarding the administration scheme. Since in clinical trials[1-4] treatment is usually given intermittently for 1 or 2 weeks for reasons of toxicity, we tested the effectiveness on tumor growth of shorter periods of treatment. We, therefore, treated the animals with the optimal drug combination 80,000 U gamma-IFN and 100 μg TNF for one day or for two weeks starting 48 hours after tumor implantation (fig. 3). A single administration of the combination already gave a clear growth inhibition. When treatment was continued for two weeks, tumors started to grow 21-58 days later than the control group (lag-period). However, all rats developed a tumor.

Figure 3

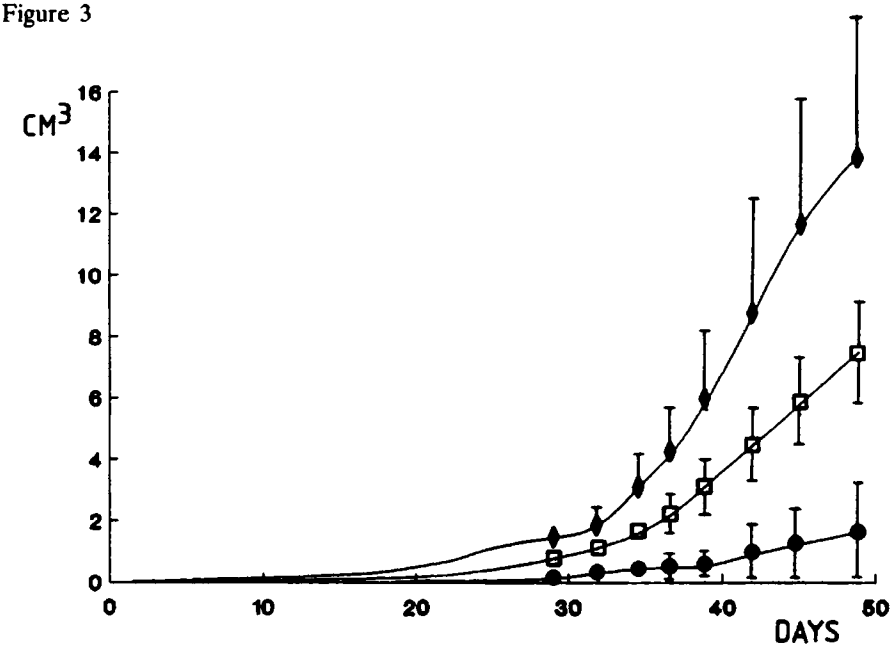


Figure 3. Effects of shorter duration of therapy with the optimal drug combination 80,000 U gamma-IFN and 100 μg TNF. Treatment started 48 hours after tumor implantation. ♦—♦ control, □—□ a single administration ($p = 0.001$) and ●—● two weeks treatment ($p = 0.002$).

Treatment does not induce immunity

To determine whether BRM therapy using the combination 80,000 U gamma-IFN and 100 μg TNF can induce specific immunity, as observed by Palladino et al[26], we rechallenged 6 cured rats with a subcutaneous tumor. 200 days after tumor implantation and subsequent treatment a new tumor piece of 50 mg was implanted in the contralateral (left) flank. All rats developed a tumor which started to grow without a lag-period and with a normal growth rate.

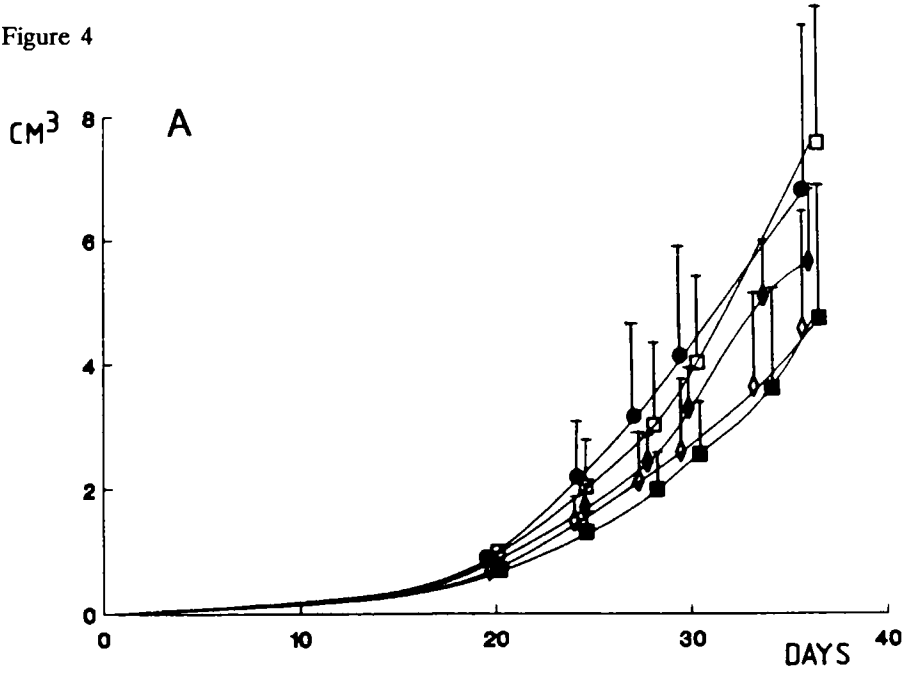
The influence of tumor volume at start of therapy

The first series of experiments revealed that gamma-IFN and TNF therapy can inhibit tumor growth completely. Whether this therapy is effective in provoking the regression of larger tumors was studied by starting therapy at different tumor volumes.

Monotherapy started at a tumor volume of 0.2-0.5 cm^3 had no effect on tumor growth (fig. 4A).

The most effective BRM combinations as established in the low tumor load studies, were tested for their antiproliferative efficacy when treatment was started at higher tumor loads. The combination with 10 μg TNF had no antiproliferative effects (fig. 4B). The combination with 100 μg TNF, however, slowed down tumor growth significantly (fig. 4C). Even when tumors reached a size of two to five cm^3, growth rate could be inhibited almost completely. However, no regression of tumor size could be provoked. After completion of therapy all tumors started to grow again with the same volume doubling time.

Figure 4



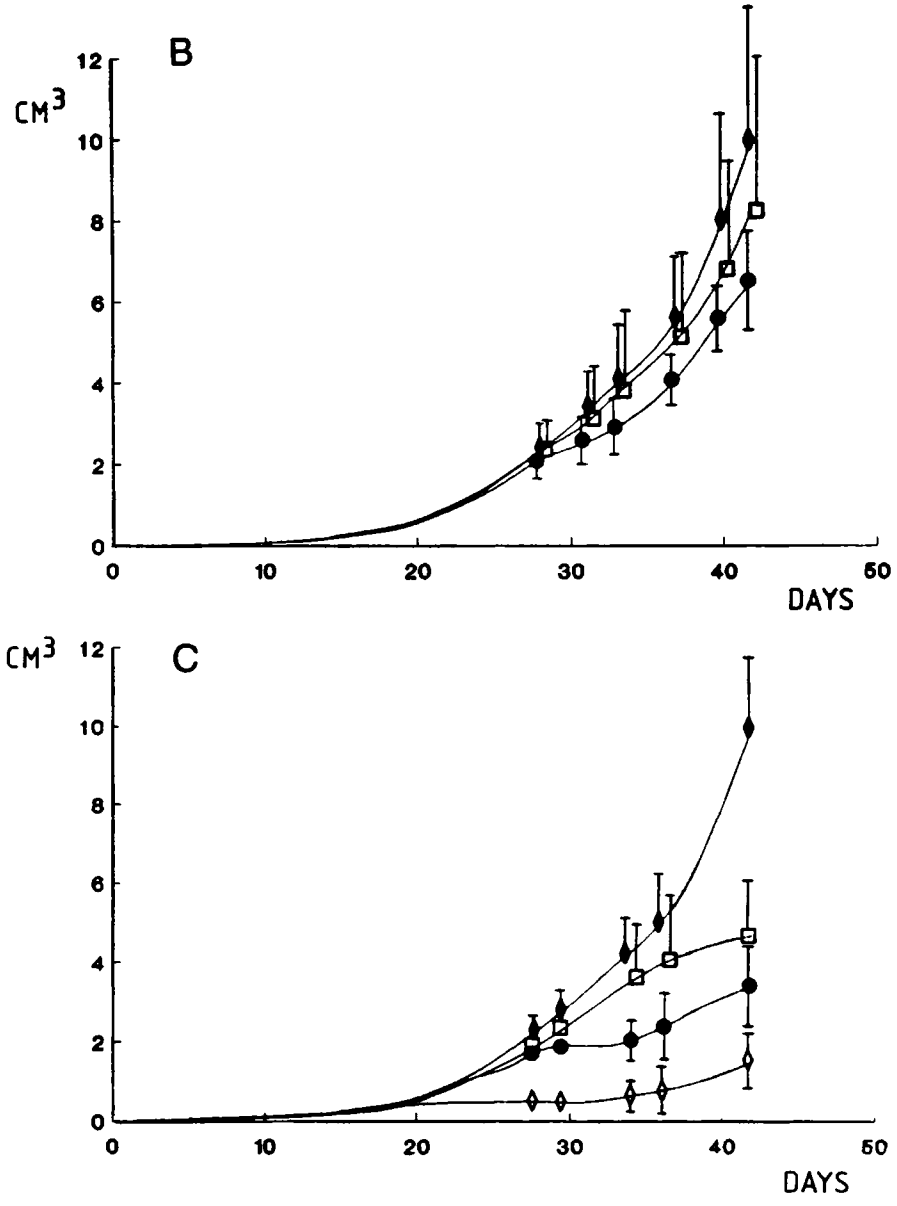


Figure 4. The influence of tumor volume at start of therapy. Gamma-IFN 3 times and TNF 5 times per week, s.c., peritumorally.

A: Monotherapy started at tumor volume of 0.2-0.5 cm^3. ♦—♦ control, □—□ 8,000 U gamma-IFN (n.s.), ●—● 80,000 U gamma-IFN (n.s.), ◇—◇ 10 μg TNF (n.s.) and ■—■ 100 μg TNF (n.s.).

B: Combination therapy with combination of 80,000 U gamma-IFN and 100 μg TNF. ♦—♦ control, □—□ start treatment at 0.2-0.5 cm^3 (n.s.) and ●—● start treatment at 1 cm^3 (n.s.).

C: Combination therapy with combination of 80,000 U gamma-IFN and 100 μg TNF. ♦--♦ control, ◇—◇ start treatment at 0.2-0.5 cm^3 ($p = 0.005$), ●—● start treatment at 1 cm^3 ($p = 0.003$) and □—□ start at 2-5 cm^3 ($p = 0.001$).

n.s. = not significant

Side effects of therapy

No obvious side effects were seen at any dose of gamma-IFN or TNF alone or in combination. No rats showed any signs of lethargy or wasting. At the end of the treatment period all rats were weighed and all groups had gained weight within the same range as shown in Table I.

DISCUSSION

Chemotherapy and radiotherapy have limited therapeutic efficacy in advanced renal cell carcinoma. Therefore, new treatment modalities must be developed. Previous in vitro studies have shown that Biological Response Modifiers such as gamma-IFN and TNF have antiproliferative effects on renal cell carcinoma[11]. In the studies presented here, we investigated the antiproliferative effects of these BRMs in a non-immunogenic animal model system.

Gamma-IFN monotherapy showed a better effect with increasing doses. These results suggest a dose-dependent relationship between gamma-IFN dose and tumor growth inhibition. Similar observations were made by Gresser[27] with mouse alpha/beta-IFN in a syngeneic Friend Leukemia tumor model system. Talmadge[15], however, showed that 50,000 U of mouse gamma-IFN 3 times per week was highly effective, whereas 100,000 U of this IFN had no therapeutic effectivity, suggesting a bell-shaped dose-response curve, i.e. a dose-optimum. It cannot be excluded that higher doses of gamma-IFN in our model system will also have less efficacy on tumor growth. TNF monotherapy was only effective with the highest dose of 100 μg. This indicates that TNF monotherapy will have very limited clinical potential, which is already apparent from phase I trials[3].

The combination of 8,000 U gamma-IFN and 10 or 100 μg TNF has additive effects, whereas combinations of 80,000 U gamma-IFN with 10 or 100 μg TNF have clearly synergistic antiproliferative effects ($p<0.001$). Animals in which no tumor growth was evident after 6 weeks of treatment, remained more than 200 days tumor free and were considered cured. In different rodent model systems, this combination has also been shown to be effective against other solid tumors, such as breast, bowel and lung carcinomas and melanomas[12-15].

Having found a drug combination that can prevent tumor formation completely after 6 weeks of treatment, we shortened the treatment period in a separate experiment. A single administration of a combination of 80,000 U gamma-IFN and 100 μg TNF resulted in a significant growth inhibition, but no lag-period was observed. Two weeks of treatment, however, prolonged the lag-period by at least 21 days. It is, therefore, conceivable that intermittent therapy, i.e. two weeks treatment, two weeks no treatment, can be sufficient for anti-tumor therapy.

Palladino et al.[26] found that TNF treatment of Meth A sarcoma bearing mice

resulted in the establishment of a tumor specific immunity. Mice which had rejected a s.c. Meth A sarcoma were immune to rechallenge with a lethal dose of this tumor 35 days after initial challenge. In contrast, specific immunity did not develop after alpha-IFN treatment of the intraperitoneally growing L1210 tumor[28]. Similarly, no specific immunity was found in our model system. Rechallenge 200 days after initial tumor implantation had no influence on tumor take or growth rate. Under these experimental conditions no specific immune response was induced by gamma-IFN and TNF treatment which suggests that a direct anti-tumor mechanism and/or macrophage and NK cell mediated tumor cell lysis are responsible for the growth inhibition. However, it cannot be excluded that the cytokines can induce tumor associated antigens necessary for a specific immune response. Further studies are needed to elucidate the working mechanisms of these BRMs.

In most clinical trials BRM treatment is started when the patient has proven (progressive) metastatic disease. The patient has a high tumor load even when a tumor nephrectomy has been performed. Therefore, we investigated the effect of gamma-IFN and TNF on higher tumor loads. Starting therapy 15 days after tumor implantation at a tumor volume of 0.2-0.5 cc, gamma-IFN or TNF monotherapies were not effective on even the smallest tumors. This is in good agreement with the limited efficacy of IFN monotherapy observed in clinical trials[2,4].

The combination of 80,000 U gamma-IFN and 10 μg TNF, which was very effective when treatment was started 48 hours after tumor implantation did not inhibit tumor growth when started at higher tumor volumes, however the combination of the highest doses of gamma-IFN and TNF significantly slowed down tumor growth, but did not provoke tumor regressions. Similar observations of the influence of tumor volume at start of therapy were elegantly shown for experimental prostatic carcinoma[29]. These experiments clearly demonstrated the need to carry out tumor debulking and adjuvant chemotherapy as early as possible in order to minimize the total tumor load and to maximize the possibility of cure.

Pilot studies showed that peritumoral injections were more effective than i.p. or i.v. treatment in our model system. In clinical trials, however, treatment is usually given systemically unequivocally resulting in lower local drug levels. Reasoning that the smallest tumors were most sensitive to low BRM doses (fig. 2 compared with figs. 4A and 4B), we might argue that patients with RCC should be treated as early as possible, as in the case of localized disease, starting with therapy immediately after nephrectomy. Due to the unpredictable clinical course of this disease a significant number of patients with stage I RCC would be overtreated which is clearly unacceptable. In addition to this therapy it is, therefore, of major importance that new diagnostic tools are developed that help us to find patients with a poor prognosis (i.e. patients at risk). Awaiting these new diagnostic tools a randomized clinical trial could be started comparing surgery alone vs. surgery plus adjuvant IFN and TNF in patients with locally advanced RCC and/or with micrometastases in the regional lymphnodes (stage II and III).

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CHAPTER III

COMBINED EFFECTS OF TUMOR NECROSIS FACTOR ALPHA AND RADIATION IN THE TREATMENT OF RENAL CELL CARCINOMA GROWN AS SPHEROIDS

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ABSTRACT

We have investigated the antiproliferative effects of Tumor Necrosis Factor Alpha (TNF) and radiation on a recently described rat renal cell tumor line grown as multicellular tumor spheroids (MTS). Treatment commenced when the spheroids had reached a diameter of 250 μm. TNF was diluted in the tissue culture medium in different concentrations, ranging from 250-1000 ng/ml. TNF monotherapy had a dose-dependent inhibiting effect on spheroid growth. Single-dose irradiation with 2, 4 or 6 Gy also retarded spheroids significantly in their growth. In the combination treatment the highest dose of TNF (1000 ng/ml) was added 4 hours prior to radiation. TNF could not induce a potentiation of the radiation injury at 2 Gy. The combination with 4 Gy, however, had additive-, and the combination with 6 Gy synergistic antiproliferative effects; in these treatment regimens respectively 2 and 5 out of 24 spheroids were controlled, i.e. cured. These experiments suggest that TNF in combination with radiotherapy may be beneficial for the treatment of renal cell carcinoma or cancer in general.

INTRODUCTION

There are currently no effective standard treatments for renal cell carcinoma (RCC). Hormonal therapy can result in a small proportion (0-10%) of objective responses, but these are incomplete and of short duration. Single or combination chemotherapy also gives no clearly definable improvement in patient survival (1).

Immunotherapy is a relatively new approach in cancer therapy. Today, interferons (IFN) are the most extensively studied Biological Response Modifiers (BRMs) in metastatic RCC. Numerous studies with natural and recombinant IFN-alpha have been conducted with an overall objective response of 15% (2).

Tumor Necrosis Factor Alpha (TNF), first described by Carswell and associates as an antitumor substance in the serum of mice treated sequentially with BCG and endotoxin (3), is a cytokine with a number of biological activities (4), particularly anti-tumor activity. In vitro and in vivo studies using RCC xenografts showed antiproliferative effects of TNF monotherapy and synergistic growth-inhibitory effects when combined with IFN (5,6,7). In a rat renal cell tumor model system TNF had a dose-dependent anti-tumor effect when treatment started 48 hours after tumor implantation, combination treatment with gamma-IFN inhibited tumor growth completely. TNF monotherapy, however, was not effective against an established tumor, i.e. when treatment started 15 days after tumorimplantation (8).

Studies using BRMs in combination with radiotherapy are rare. The combination of Interleukin-2 with Tumor Infiltrating Lymphocytes and radiotherapy produced synergistic effects against murine sarcomas (9). TNF augmented the radioresponse of a murine mammary carcinoma in vivo but not in vitro (10,11). These studies indicate that combination of immunotherapy and external beam irradiation can be useful in cancer treatment.

Multicellular tumor spheroids (MTS) are an in vitro model of intermediate complexity between tumors in vivo and conventional in vitro culture systems. MTS represent small avascular tumors or metastases (12,13,14). MTS in tissue culture have almost spherical geometry, with an outer layer mainly consisting of dividing cells and an intermediate layer of resting cells. After a certain diameter, different in each type of spheroids, has been reached, necrotic regions develop in the centre. In the present study we report on the responses to treatment of rat renal tumor spheroids with TNF or radiotherapy and their combinations.

MATERIAL AND METHODS

Tumor cells

The HM carcinoma cell line is derived from a spontaneously arisen rat renal cell tumor (15,16). Cells were maintained in complete medium consisting of RPMI 1640 culture medium supplemented with 10% foetal calf serum, 2mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (GIBCO Ltd, Paisley, Scotland), 250 nM dexamethasone and 20 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (Sigma, St. Louis, MO, USA). Stock cultures of the cells were maintained in monolayer culture at 37 C in 6% CO_2 in air with 100% humidity.

Spheroids

The methods of producing spheroids have been described elsewhere (17). In short: single cell suspensions were obtained by trypsinizing exponential monolayer cultures and inoculating 1 x 10^5 cells into 60 mm Petri-dishes (Nunc AS, Kastrup, Denmark) coated with 1% agarose (BDH Chemicals Ltd, Poole, England) to prevent attachment to the dishes. After four days of incubation, small spheroids (200 μm) could be harvested and were transferred to individual wells of 24-wells test plates (Costar, Cambridge, MA, USA) coated with 1% agarose. Plates were incubated as above with weekly changes of medium.

TNF-treatment

Recombinant human TNF was generously supplied by Boehringer Ingelheim (Alkmaar, The Netherlands). The specific activity of TNF determined in the presence of Actinomycin D was 6 x 10^7 Units/mg protein as determined in the L-929 cytotoxicity assay. The purity was >99% as determined by SDS polyacrylamide gel electrophoresis and it contained less than 1.0 ng endotoxin per mg. protein based on the limulus amoebocyte assay. The original TNF solution was diluted with RPMI 1640 culture medium to a final concentration of 50.000 ng/ml, divided in aliquots and stored at -80 C. Treatment started when spheroids reached a diameter of 250 μm. Immediately prior to use the TNF solution was further diluted with complete RPMI 1640 culture to final concentrations of 250, 500 and 1000 ng/ml. Each well contained 1 ml medium and was changed biweekly. Control spheroids received complete medium only.

Irradiation

Spheroids were irradiated at a diameter of 250 μm using a 18 MV photon beam from a Saturne linear accelerator (CGR, Buc, France) at a dose-rate of 2 Gy/min, with a wax build-up to ensure homogeneous energy deposition within each well. Radiation was applied as a single dose. Control plates were removed from the incubator for the same period of time (20-40 min). In the combined treatment

modality studies, the TNF containing medium was given 4 hours before irradiation. Spheroids were irradiated with the drug in the medium.

Treatment responses

The growth of spheroids on test plates was determined by measurement of diameters of individual spheroids using a calibrated scale in the eye-piece of an inverted microscope two times per week. Assuming spherical geometry, volumes were calculated and growth curves constructed by plotting the ratio log (volume/initial volume) as a function of time after treatment. Growth delay (GD) was estimated from the difference in time required for non-treated and treated spheroids to reach a volume of five times the original volume. To correct for possible differences in growth rate, the specific growth delay (SGD) was calculated as: SGD = GD/V_D, where V_D is the volume doubling time of untreated control spheroids.

RESULTS

Spheroids of a rat renal cell tumor line grown in vitro, were used to test the direct antitumor effects of TNF and/or radiation.

Antitumor activity of TNF monotherapy

Spheroids of 250 μm diameter were incubated with increasing doses of TNF (250-1000 ng/ml culture medium). Freshly diluted TNF in complete medium was changed twice weekly. TNF monotherapy was started at day 0. To evaluate the effectiveness of different treatments the difference in time to reach a size 5 times the initial volume was measured, i.e. growth delay (GD). To compare results between different experiments the specific growth delay (SGD) was calculated by dividing GD with the volume doubling time of the respective untreated growth control. Under the experimental conditions described, TNF has an antiproliferative effect with the highest dose resulting in the strongest growth inhibition, indicating a dose-dependent response (figure 1). SGD increased from 1.4 with the lowest dose to 3.5 with the highest dose of TNF (table I).

Effects of radiation alone

To see whether radiation alone could inhibit spheroid growth, we exposed spheroids to graded doses (2-6 Gy) of radiation (fig. 2). Irradiation produced retardation of growth, for varying time periods, depending on the radiation dose. The curves for the 2 and 4 Gy treated groups showed identical effects on day 4. The effect for 4 Gy treated spheroids increased until day 7, after which growth was resumed at a doubling time comparable to the 2 Gy treated and the control group. The growth inhibitory effect after 6 Gy was significantly higher than in the 2 and 4

Gy treated groups. This effect was already evident at day 4, and persisted until day 12, when growth was resumed at approximately control rate. The lateral displacement (growth delay) of the growth curves after irradiation is not linear proportional to the dose, suggestive of a non-linear dose-response relationship.

Figure 1

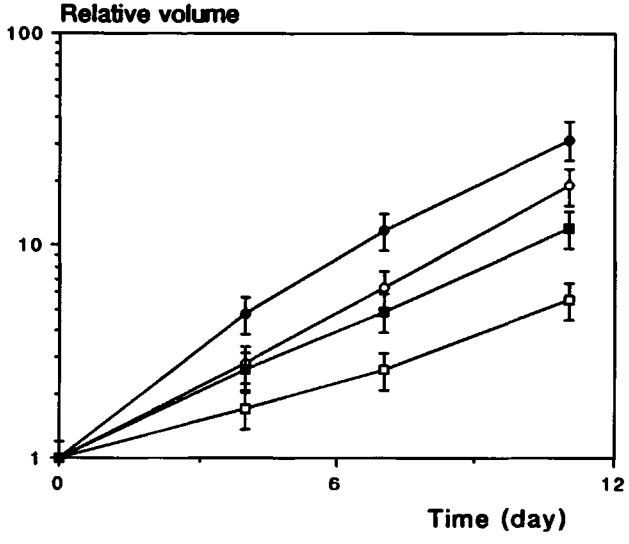

Figure 1. Effect of TNF monotherapy on spheroid growth. The Y-axis represents the relative volume plotted on a logarithmic scale as log (volume/initial volume). Each point represents the average volumes of at least ten spheroids. Bars represent standard errors. ●—●, control, 0—0, 250 ng/ml TNF, ■—■, 500 ng/ml, □—□, 1000 ng/ml.

Table I. Specific growth delay (SGD) in HM spheroids induced by different treatments

Treatment	Dose	S.G.D. ± SD[1]
TNF	250 ng/ml	1.4 ± 0.3
TNF	500 ng/ml	1.9 ± 0.5
TNF	1000 ng/ml	3.5 ± 0.5
radiation	2 Gy	1.3 ± 0.2
radiation	4 Gy	1.8 ± 0.4
radiation	6 Gy	5.2 ± 0.5
combination	TNF 1000 ng/ml + 2 Gy	3.4 ± 0.3
combination	TNF 1000 ng/ml + 4 Gy	5.2 ± 0.5
combination	TNF 1000 ng/ml + 6 Gy	12.4 ± 0.7

[1]SD means standard deviation

Figure 3

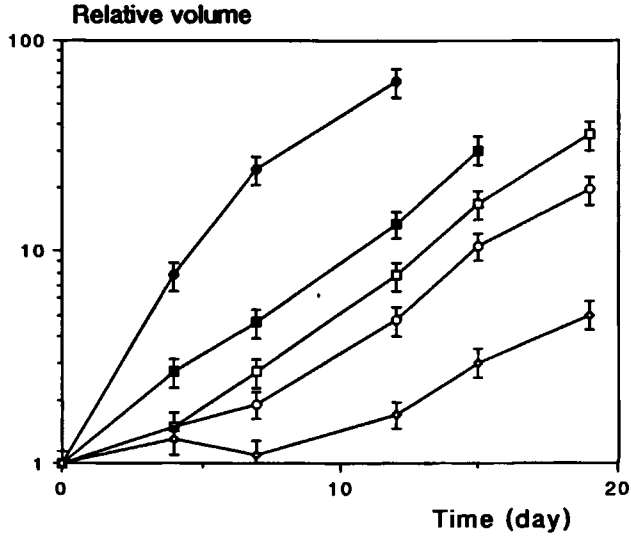

Figure 3. Growth curves for spheroids treated with the combination of 1000 ng/ml TNF and radiation. Spheroids that regressed completely following treatment were not included in the curves. The Y- axis represents the relative volume plotted on a logarithmic scale as log (volume/initial volume). Each point represents the average volumes of at least 19 spheroids. Bars represent standard errors. ●—●, control, ■—■, 1000 ng/ml TNF monotherapy, □—□, TNF + 2 Gy, ○—○, TNF + 4 Gy, ◇—◇, TNF + 6 Gy.

DISCUSSION

Advanced RCC is generally resistant to medical treatment. Neither chemo- nor hormonal therapy have improved patient survival, therefore new treatment modalities must be developed. BRMs, like IFNs and interleukins, showed promising results in phase I and II studies (19,20). Phase I studies indicated that TNF treatment is feasible (21). If TNF will be used in cancer treatment, it is likely to be used in combination treatments, e.g. in combination with IFNs (22). In the present study we tested whether TNF might improve tumor radioresponse.

We used multicellular tumor spheroids (MTS), a three-dimensional in vitro tumor model, that resembles the in vivo tumor situation more closely than conventional monolayers do. Mueller-Klieser already suggested that TNF should be tested in the spheroid system (13), but to our best knowledge this has not been done so far.

TNF monotherapy showed a dose-dependent antiproliferative effect. This is in accordance with other in vitro studies which showed the direct antiproliferative effects of TNF against numerous tumor cell lines (23). Radiation alone also produced a dose-dependent growth retardation. Combination treatments using the

Figure 2

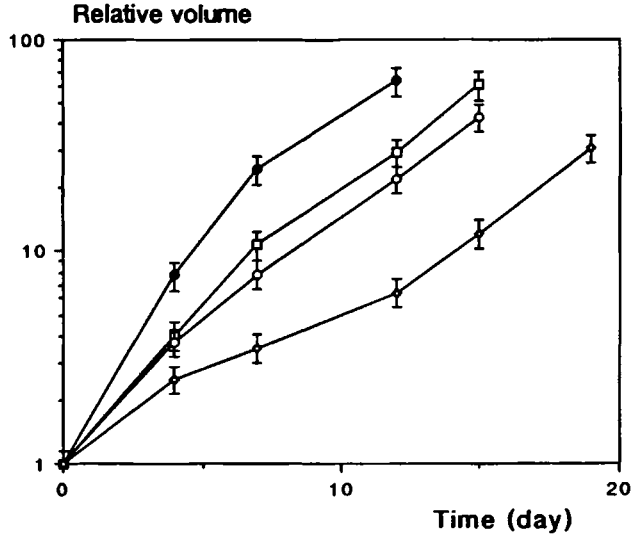

Figure 2. Effect of single doses of 18 MV X rays The Y-axis represents the relative volume plotted on a logarithmic scale as log (volume/initial volume) Each point represents the average volumes of at least 20 spheroids Bars represent standard errors ●—●, control, □—□, 2 Gy, ○—○, 4 Gy, ◇—◇, 6 Gy.

TNF combined with radiation

The highest dose of TNF was used to assess the effects of combining both therapies. Spheroids were irradiated with different doses in the presence of 1000 ng/ml TNF. At day 0, 4 hours prior to irradiation TNF was added by changing media. The combination of TNF with 6 Gy showed the largest effect; after an initial regression of volume, finally most spheroids started to regrow with the same growth rate as the untreated controls. 2 and 5 out of 24 spheroids, however, failed to regrow and were considered controlled or 'cured' in the combination treatments with 4 and 6 Gy, respectively.

A combination of two treatments is considered additive when the effect of the combination is equal to the sum of the two separate treatments, and considered synergistic when the effect of the combination is greater than the sum of the two separate treatments (18). Table 1 summarizes the growth delay values derived from the growth curves. 2 Gy showed no potentiating effect on TNF-therapy. 4 Gy, however, has an additive effect when combined with TNF, and the combination of TNF with a radiation dose of 6 Gy clearly showed synergistic effects, since the SGD of the combination, 12.4, is greater than 8.7, the addition of the two single treatments (3.5 for TNF 1000 μg/ml plus 5.2 for 6 Gy).

most effective TNF-dose plus radiation produced a greater effect than the respective monotherapies, as manifested by an increased growth delay. The combination with 4 Gy has additive, while that with 6 Gy has synergistic antiproliferative effects. Whereas neither TNF nor irradiation alone produced permanent spheroid control, 2 and 5 out of 24 spheroids were controlled, i.e. cured, in the groups that received TNF plus 4 and 6 Gy, respectively.

Spheroids serve as an in vitro model for the early avascular stage of tumor growth, where the degree of structural and functional differentiation in the primary tumor may be retained. In vivo studies using the original rat renal cell tumor of our HM cell line revealed that TNF treatment was not effective when treatment started 15 days after tumor implantation, i.e. a very small palpable tumor (8). Our data suggest that inclusion of radiation in our therapeutic regimens can enhance the anti-tumor effects of TNF. Studies with a murine mammary carcinoma revealed a significant increase of tumor radioresponse when TNF was combined with local radiotherapy (10).

Several other BRMs, like IFN, interleukin-2 (IL-2) and BCG, have been investigated for their ability to improve radiation response. In vitro studies showed that IFNs potentiated radiation cytotoxicity (24), this phenomenon is associated with an increased blockage at G2-M phase in the cell cycle (25). IL-2 alone could not increase mean survival time of mice with experimental hepatic metastases (11). Addition of local liver radiation prolonged survival time significantly, best results were obtained with triple treatment of radiation, IL-2, and Tumor Infiltrating Lymphocytes. Total body irradiation, however, completely abrogated the effects of IL-2 due to immunosuppression. Bacillus Calmette Guerin (BCG) also induced a significant decrease in tumor growth in association with local tumor irradiation (26,27). The timing of BCG administration appeared to be an important factor, BCG therapy begun either before or immediately after irradiation was effective, whereas delay of BCG treatment to 4 days after irradiation showed no significant difference with the control group, which received radiotherapy alone.

TNF has pleiotropic effects, e.g. it can induce procoagulant formation by endothelial cells and stimulate increased levels of fibrinogen in circulation, which will promote the formation of microthrombi in tumor vasculature (4). The hypoxic conditions created in such an environment protect tumor cells from radiation damage. TNF also prompts endothelial cell to production of IL-1. IL-1 is considered a radioprotector since pretreatment with IL-1 protects mice in a dose-dependent manner from the lethal effects of radiation (28). The best timing for TNF administration seems to be immediately after radiotherapy. Sersa et al. (10) and Nishiguchi et al. (11) gave TNF 3 hours after irradiation and showed synergistic effects. General conclusions about the administration scheme cannot be drawn from these studies since they did not include a treatment group with TNF therapy prior to radiotherapy in their protocols.

In conclusion, administration of TNF as an anti-tumor agent is capable of augmenting radioresponse of a rat renal cell carcinoma grown as spheroids. More studies are needed to assess the therapeutic scope and potency of TNF when combined with radiotherapy in the treatment of RCC and cancer in general.

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CHAPTER IV

COMBINATION TREATMENT WITH TUMOR NECROSIS FACTOR ALPHA AND DNA TOPOISOMERASE-II-TARGETED DRUGS IN A RAT RENAL CELL CARCINOMA MODEL SYSTEM

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ABSTRACT

The antiproliferative effects of two DNA topoisomerase-II targeted drugs, Adriamycin and VP16, in combination with recombinant human tumor necrosis factor alpha (TNF), were investigated in a rat renal cell tumor model system. In vitro studies using the double layer soft agar system were performed with the HM cell line, derived from the original rat renal cell tumor. TNF monotherapy had dose-dependent growth inhibiting effects in the dose-range tested (1 to 1000 ng/dish). Adriamycin, in doses of 200 to 20,000 ng/dish, completely suppressed colony formation. VP16, however, was less effective and could not completely inhibit colony formation (dose range 1 to 10,000 ng/dish). Different combinations of VP16 and Adriamycin with TNF had significant antiproliferative effects. Adriamycin 2 ng/dish in combination with TNF 1000 ng/dish was the only combination which resulted in synergistic effects.

For in vivo studies TNF was administered subcutaneously, peritumorally, 5 times per week. Adriamycin and VP16 were administered intraperitoneally in different treatment schedules. Treatment started 48 hours after tumor implantation. Adriamycin 1 mg/kg for three consecutive days every two weeks had significant antitumor effects, and induced synergistic antitumor effects in combination with 10 or 100 μg TNF per rat. TNF and Adriamycin mono- or combination therapy were not effective when treatment started at a tumor volume of 0.2-0.5 cm^3. VP16 monotherapy did not produce significant antitumor effects, and combination with TNF did not show substantial augmentation of the therapeutic efficacy.

INTRODUCTION

Renal cell carcinoma (RCC) is the most common kidney tumor in adults. Approximately 25-30% of patients with RCC have metastases at time of diagnosis (deKernion and Mukamel, 1987). Furthermore, metastases will develop in a majority of untreated patients within one year after discovery. Systemic therapy with hormonal therapy or chemotherapy has not significantly improved the prognosis of patients with disseminated disease (Harris, 1983; Yagoda, 1989). Objective responses using hormonal therapy range from 0 to 10 %, with nearly all responses being partial and of short duration. Numerous chemotherapeutic agents in mono- or combination therapy have been administered in clinical trials, the overall response rate in 2,120 patients was 8.77%. Vinblastin monotherapy is the most effective agent, however, efforts to improve its response rate by adding other chemotherapeutic agents have been unsuccessful.

Immunotherapy with cytokines is a relative new approach to the treatment of cancer (Foon, 1987; Borden and Sondel, 1990). Interferon (IFN) is the most extensively studied cytokine in RCC and is associated with approximately 16% objective responses (Horoszewicz and Murphy, 1989; Heicappell and Ackermann, 1990). Tumor necrosis factor alpha (TNF) is another cytokine with in vitro antitumor effects against experimental renal tumors (Beniers *et al.*, 1989; Kavoussi *et al*, 1989). TNF is synthesized by various activated phagocytic and non-phagocytic cells, including macrophages and monocytes. Furthermore, TNF can mediate the necrosis of subcutaneously implanted tumors (Carswell *et al.*, 1975; McIntosh *et al.*, 1990). The neovasculature of transplanted and spontaneous tumors in mice is specifically affected by a single dose of TNF (5-20 μg/mouse), but there is no effect on the vascularization of normal tissue transplants. Growth of various tumors can be inhibited in syngeneic tumor models and in human tumor xenografts in nude mice (Haranaka *et al.*, 1984; Krosnick *et al.*, 1989; Baisch *et al*, 1990;Van Moorselaar *et al.*, 1990). Although combinations of IFN and TNF can result in the enhancement of their respective antitumor activities in experimental models (van Moorselaar *et al.*, 1990; Beniers *et al.*, 1991), clinical trials are not conclusive yet. One complete remission was found in 14 patients evaluable for response in a phase II trial with IFN-gamma and TNF (de Mulder *et al.*, 1991), whereas preliminary results of a study with IFN-alpha and TNF showed 43% objective responses among 14 patients (Otto *et al.*, 1990).

The combination of chemotherapy with immunotherapy seems very promising (Mitchell, 1988; Wadler and Schwartz, 1990). Clinical trials with IFN-alpha and vinblastin produced objective responses in 31% and 34% of patients with RCC (Fossa *et al.*, 1986; Bollack *et al.*, 1991). Another study, however, could not confirm these results with 16% objective responses (Schornagel *et al.*, 1989). Marked enhancement of in vitro cytotoxicity has been demonstrated when TNF is added to

cultures of murine L929 fibrosarcoma and MBT-2 transitional cell carcinoma cells coincident to or following the addition of topoisomerase-II-targeted drugs (Alexander *et al.*, 1987a; Baloch *et al.*, 1990; Utsugi *et al.*, 1990). Human lung cancer cells, however, were resistant to these combinations (Giaccone *et al.*, 1990). DNA topoisomerase II is a homodimeric enzyme which catalyzes transient breakage and reunion of single and double strand DNA, thereby allowing strand passage of another DNA segment (Liu, 1989). This activity is required for DNA synthesis, chromosome condensation during mitosis and for gene transcription. Topoisomerase II has been identified as the site of action for some clinically used chemotherapeutic drugs, including Adriamycin, Actinomycin D, VP16 and VM26. These drugs can be broadly categorized into DNA intercalating drugs, such as Adriamycin, and non-intercalating drugs such as VP16. Both categories of drugs stabilize the transient cleavable complex of the enzyme which becomes covalently bound to the 5' terminus of DNA during the breaking-reunion reaction, resulting in increased DNA excision. This is easily detectable by alkaline eluation as DNA single or double strand-breaks and DNA-protein cross-links (Liu, 1989; Binaschi *et al.*, 1990; Branellec *et al*, 1990). In vivo studies with a weakly immunogenic 3-methyl cholanthrene-induced sarcoma (MCA-106) showed synergistic effects of Adriamycin in combination with TNF (Krosnick *et al*, 1989). VP16 also produced synergistic effects in concert with TNF against two RCC xenografts and in the syngeneic murine bladder tumor MBT-2 model (Alexander *et al.*, 1987b; Burgers *et al.*, 1989; Donaldson *et al.*, 1990).

In the study presented here we have tested the in vitro and in vivo antiproliferative effects of Adriamycin and VP16 in combination with TNF in a syngeneic rat renal cell tumor model system which histologically and biochemically closely resembles human RCC (deVere White and Olsson, 1980). Adriamycin could induce synergistic effects, whereas VP16 was not effective in this respect.

MATERIAL AND METHODS

Animals

Adult male Lewis rats, weighing 330-350 gram (Charles River Wiga GmbH, Sulzfeld, FRG), were kept 3 rats per cage with a regimen of 12 hours light per day. The animals were allowed access to RMH-TM food (Hope Farms BV., Woerden, The Netherlands) and water ad libitum.

Tumor Line

The rat renal cell tumor line was kindly provided by Dr. R. Babayan, Boston, USA. The tumor was maintained by serial subcutaneous transplantation. For in vivo experiments, the tumor was excised from a tumor-bearing animal, and after

removal of normal and necrotic tissue, the tumor tissue was cut into pieces of 40 mg (38-43 mg). The precise weight of each tumor piece was measured on preweighed sterile gauzes on a laboratory scale (Mettler). An 0.5 cm incision was made in the right flank of recipient animals, and after separating the subcutaneous tissue by blunt dissection, the tumor fragment was placed subcutaneously (s.c.) . The incision was closed using skin clips (7.5 x 1.75 mm, Aesculap, Germany). In this way a tumor take of 100% was achieved.

The HM carcinoma cell line is derived from the rat renal cell tumor (van Moorselaar 1991, complete characterization will be described elsewhere). Cells were maintained in complete medium consisting of RPMI 1640 culture medium supplemented with 10% foetal calf serum, 2mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (GIBCO Ltd, Paisley, Scotland), 250 nM dexamethasone and 20 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (Sigma, St. Louis, MO, USA). Stock cultures of the cells were maintained in monolayer culture at 37 C in 6% CO_2 in air with 100% humidity. The HM cell line is tumorigenic in Lewis rats and has a growth rate similar to that of the original tumor.

In vitro testing with the double layer soft-agar assay.

Single cell suspensions were obtained after quick trypsinization of subconfluent monolayer cultures. Cell density and viability were determined by adding 15 μl trypan blue solution (25 mg in 5 ml 3% acetic acid) to 15 μl cell suspension and simultaneously counting colored and non colored cells using a Bürker Türk haemocytometer. Suspensions with a viability of 95% or more were used for in vitro testing. For the detection of the in vitro growth potential a modified double layer soft agar culture method as originally described by Salmon and Hamburger was used. An underlayer of one ml 0.5% agar in double enriched (DE) McCoy's 5A medium was plated in 35 mm dishes and allowed to gel at room temperature. Tumor cell suspensions were plated in the upper layer consisting of DE-CMRL 1066 medium with 0.3% agar. In a pilot study we plated a range of 10,000 to 100,000 cells of the HM cell line. The optimal starting concentrations appeared to be 50,000 cells per dish, resulting in approximately 900 colonies after 14 days of culture, i.e. plating efficiency of 1.8%. Cells were cultured immediately after preparation of the single cell suspension. Dishes were incubated at 37 C in 6% CO_2 in air with 100% humidity. Growth potential was quantified using an Omnicon Fas II automated colony counter (Milton Roy Inc., Rochester, New York). Results are presented as the growth potential of cells relative to the untreated control cells.

Drugs

Recombinant human TNF alpha was kindly provided by Boehringer Ingelheim (Alkmaar, The Netherlands). The specific activity of TNF determined in the presence of Actinomycin D was 6 x 10^7 Units/mg protein as determined in the

L-929 cytotoxicity assay. The purity was >99% as determined by SDS polyacrylamide gel electrophoresis and it contained less than 1.0 ng endotoxin per mg protein based on the limulus amoebocyte assay. The original TNF solution was diluted with RPMI 1640 culture medium (Gibco, Paisley, UK) to a final concentration of 2, 20, or 200 μg per ml, divided in aliquots and stored at -20^{0} C.

Adriamycin (Adriablastina) was obtained from Farmitalia Carlo Erba (Rotterdam, The Netherlands) and reconstituted with sterile water to a final concentration of 9, 3, 1 or 0.1 mg/ml. VP16 (Vepesid) was obtained from Bristol-Myers B.V. (Weesp, The Netherlands) and diluted with 0.9% sodium chloride to final concentrations of 20, 10, 5, or 1 mg/ml. Both drugs were used immediately after reconstitution or dilution.

Treatments and monitoring

In vitro drug tests were performed by continuous exposure of the drugs to the tumor cells. Stock solutions TNF were diluted with DE-CMRL 1066 medium to concentrations of 50, 500 or 5,000 U or ng/ml. Adriamycin or VP16 were diluted with sterile water and 0.9% sodium chloride respectively to concentrations of 10 to 100,000 ng/ml for Adriamycin and 5 to 50,000 ng/ml for VP16 and layered over the gelled upper layer in a volume of 200 μl. All agents were used immediately after dilution since chemotherapeutic agents can loose their activity by storage (Yang and Drewinko, 1985). Dishes in the control group received 200 μl DE-CMRL 1066 medium only. As a cytotoxic control we used 0.37 mM $HgCl_2$. In vitro tests were performed in triplicate and repeated at least twice.

For in vivo studies TNF was administered s.c., peritumorally, 5 times per week in a 0.5 ml injection volume. Adriamycin and VP16 were injected intraperitoneally in different treatment schedules (see Results), the appropriate dilution was chosen in such a way that all rats received an volume of 0.33 - 0.35 ml. Phosphate buffered saline (PBS) was used for the control group. Treatment started two days after tumor implantation or when the tumor reached a volume of 0.2-0.5 cm^3 and was continued until the tumors in the control group reached a volume of 10 cm^3. All experiments were carried out at least in duplicate.

Tumor growth was followed by measurements of tumor size according to the method of Janik et al. (1975) by the same measurer throughout each experiment. Three times per week length (l), width (w) and height (h) were measured using a slide caliper. No correction was made for skin thickness. Tumor volume (V) was calculated from the formula $V = 0.5236 \times l \times w \times h$.

Statistical analysis

Statistical analysis of the in vitro data was done by two-way analysis of variance on the logarithms of the colony number at day 14 after seeding. Differences between mean colony numbers were tested for significance using the

Tukey studentized range test at an experimental 0.05 error rate.

For each rat, the tumor volume was estimated on the last treatment day by loglinear regression on the tumor volumes throughout the treatment period. Analysis of variance was used to compare the final volumes of the treatment versus the control groups and to test for additive effects in the combination treatments. To test the significance of differences between groups the Tukey studentized range test was used at an experimental 0.05 error rate. 6 animals were included in each group.

The effect of treatment A is defined as $E_A = V_{control}/V_A$ and the effect of treatment B as $E_B = V_{control}/V_B$, where V_A, V_B, and $V_{control}$ are the estimates for the tumor volumes of treatment A, B or control group respectively. Additivity is defined (van Moorselaar *et al.*, 1990) as $E_{A+B} = E_A \times E_B$ (i.e. $\log E_{A+B} = \log E_A + \log E_B$). A combinatjon of two treatments is synergistic when the effect of the combination (E_{A+B}) is significantly greater than the product of the effects of the separate treatments.

RESULTS

In vitro studies

50,000 cells of the HM cell line, derived from the rat renal cell tumor, were plated in the upper layer of the double-layer soft-agar culture system. Preliminary studies with Adriamycin monotherapy revealed that doses of 200 to 20,000 ng/dish completely suppressed colony formation. We therefore used in the combination treatments the lowest doses of 2 and 20 ng/dish. VP16 monotherapy gave rise to significant growth inhibition with both highest doses of 1,000 and 10,000 ng/dish. Complete inhibition of colony formation, however, was not achieved (growth fraction 0.10 and 0.12 respectively). For combination studies we used the three lower dose levels, 1 to 100 ng/dish. Table I demonstrates the effects of Adriamycin, VP16 and TNF and their combinations as relative growth potential, i.e. number of colonies in treated dishes divided by the number of colonies in control dishes after 14 days of culture. TNF 1000 and 100 ng/dish produce significant antiproliferative effects. Adriamycin 20 ng/dish also has a significant growth inhibiting effect, whereas none of the VP16 doses produced a significant antitumor effect. Different combinations of TNF and Adriamycin 2 ng/dish or VP16 10 or 100 ng/dish show significant antiproliferative effects when compared to the untreated growth control. Moreover, the combination of Adriamycin 2 ng/dish and TNF 1000 ng/dish resulted in a synergistic effect. The combination of 2 ng/dish Adriamycin and 1 ng TNF per dish was also significantly better than the respective monotherapies. Adriamycin 20 ng/dish monotherapy is already too active to show synergism in combination with TNF. None of the combinations of VP16 and TNF produced synergistic effects.

Table I

	0	TNF 1000[a]	TNF 100	TNF 10	TNF 1
0	1.00±0.07[b]	0.56±0.02[d]	0.57±0.07[d]	0.63±0.04	0.79±0.03
Adriamycin 20[c]	0.05±0.02[d]	0.07±0.02[d]	0.06±0.02[d]	0.05±0.02[d]	0.06±0.02[d]
Adriamycin 2	0.73±0.03	0.29±0.03[d,e]	0.43±0.06[d]	0.43±0.06[d]	0.55±0.09[d]
VP16 100	0.78±0.11	0.51±0.08[d]	0.71±0.14[d]	0.61±0.04[d]	0.81±0.11
VP16 10	1.23±0.08	0.64±0.08[d]	0.74±0.06	0.93±0.06	1.14±0.05
VP16 1	1.07±0.09	0.83±0.06	0.89±0.04	0.99±0.08	1.09±0.07

[a] TNF dose in ng per dish
[b] relative growth potential, i.e. number of colonies in treated dishes divided by number of colonies in control dishes after 14 days of culture.
[c] Adriamycin or VP16 in ng per dish
[d] significantly different colony number compared to the untreated control ($p < 0.05$)
[e] synergistic combination

Table I. Inhibition of colony formation by Adriamycin, VP16 or TNF and their combinations.

TNF monotherapy in vivo

In previous studies with this rat renal cell tumor model system we evaluated the influence of administration route on the antitumor effects of TNF and rat-gamma-IFN (van Moorselaar *et al.*, 1990). Comparison of the intravenous, intraperitoneal (i.p.) and subcutaneous (s.c.) route showed that s.c. injected cytokines were the most effective. We, therefore, administered in the in vivo experiments described here TNF s.c., peritumorally. As shown before (van Moorselaar *et al.*, 1990), TNF 10 μg per rat, 5 times per week, had no significant antitumor effects, whereas a ten times higher dose could reduce tumor growth significantly (fig. 1A and 5A).

Antitumor effects of Adriamycin monotherapy or in combination with TNF

In dose-finding studies we gave Adriamycin every three weeks, either one administration of 9 mg/kg or 1 to 3 mg/kg for three consecutive days. All regimens started 48 hours after tumor implantation. Rats treated with 9 mg/kg died within 14 days after start of therapy, likewise rats treated with 3 mg/kg died within 21 days. 1 mg Adriamycin per kg every three weeks was not lethal, but did not produce significant antitumor effects (fig. 1B). Combinations of this dosage of Adriamycin and TNF 10 or 100 μg per rat also showed neither significant, nor synergistic antitumor effects (fig. 1B).

Figure 1

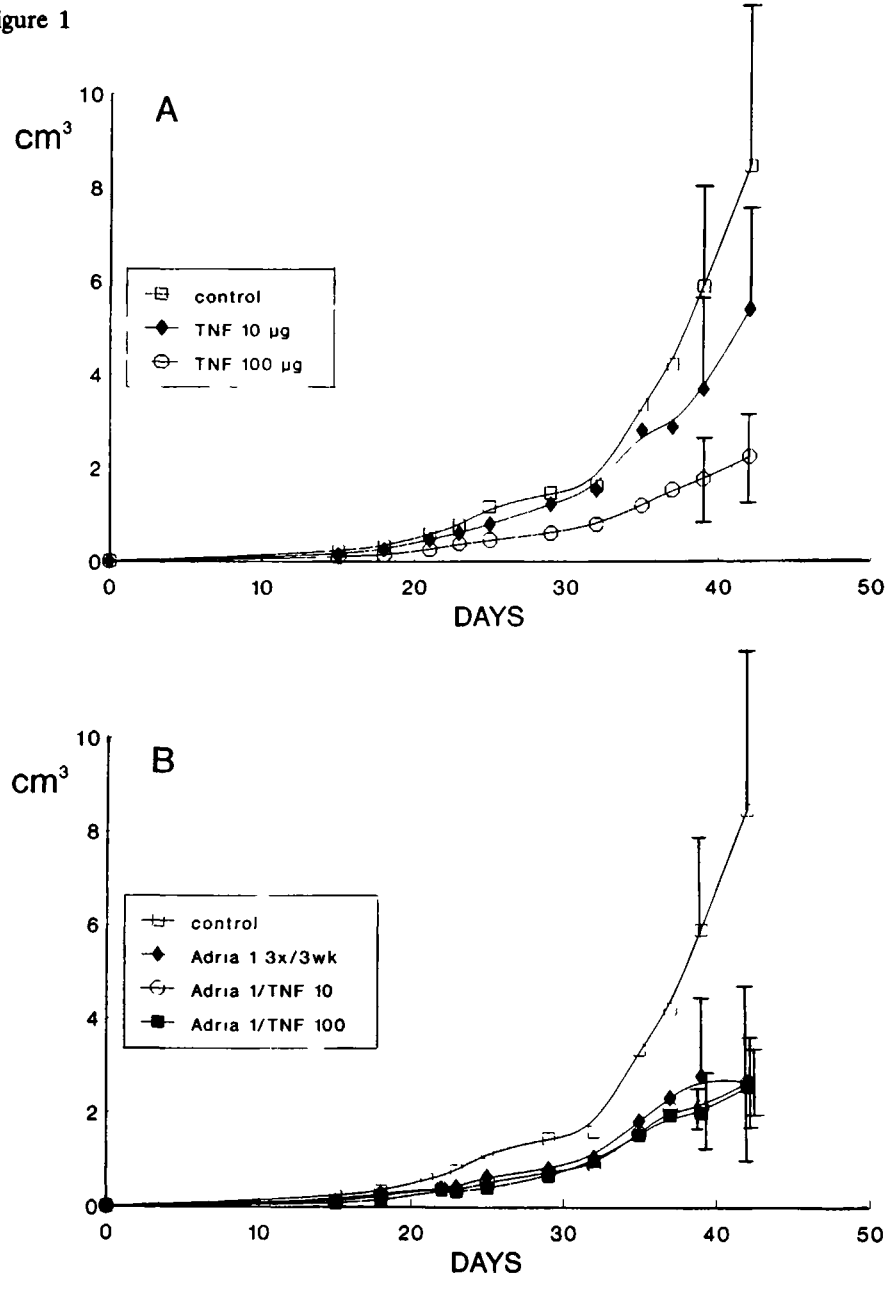
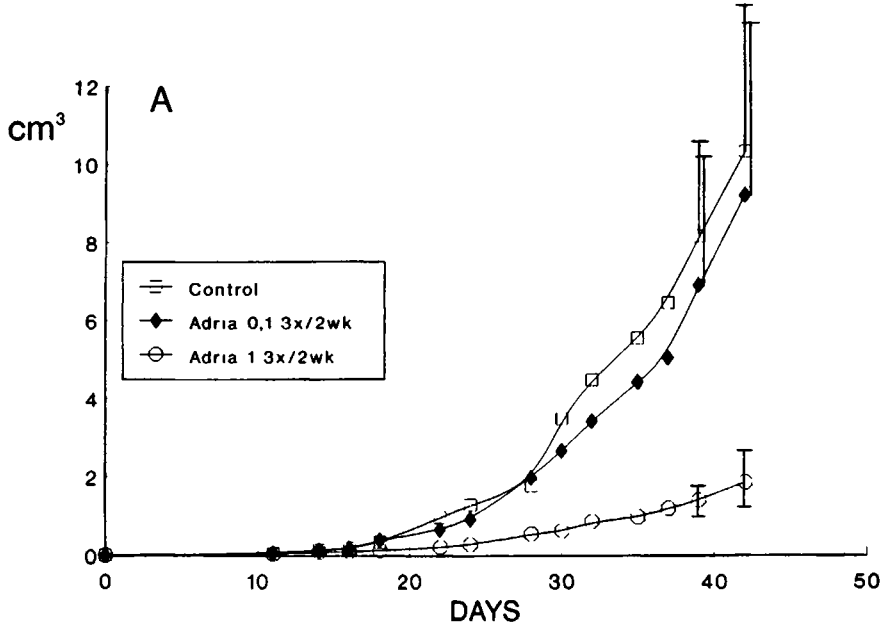


Figure 1. Antitumor effects of Adriamycin and TNF against the s.c. implanted rat renal cell carcinoma. Treatment started 48 hours after tumor implantation. A: TNF monotherapy administered s.c., peritumorally, 5 times per week. TNF 100 µg per rat has statistically significant antitumor effects. B: Adriamycin monotherapy and in combination with TNF, administered i.p. for three consecutive days every three weeks.

In order to improve the antitumor effects of Adriamycin the number of administrations were increased to three consecutive days every second week, starting 48 hours after tumor implantation. Adriamycin 1 mg/kg had significant antitumor effects, whereas a ten times lower dose was not effective in inhibiting tumor growth (fig. 2A). TNF 100 μg in combination with 0.1 mg Adriamycin per kg substantially augmented the antitumor effects, which resulted in a significant growth inhibition (fig. 2B). Both doses of TNF in combination with 1 mg Adriamycin per kg also significantly inhibited tumor growth, but more importantly these combinations had synergistic antitumor effects. Increase of the number of Adriamycin administrations to 5 times every week 0.1 mg/kg did not result in improvement of the antitumor effects. Monotherapy and combination treatment showed no significant tumor growth inhibition (fig. 3).

Figure 2



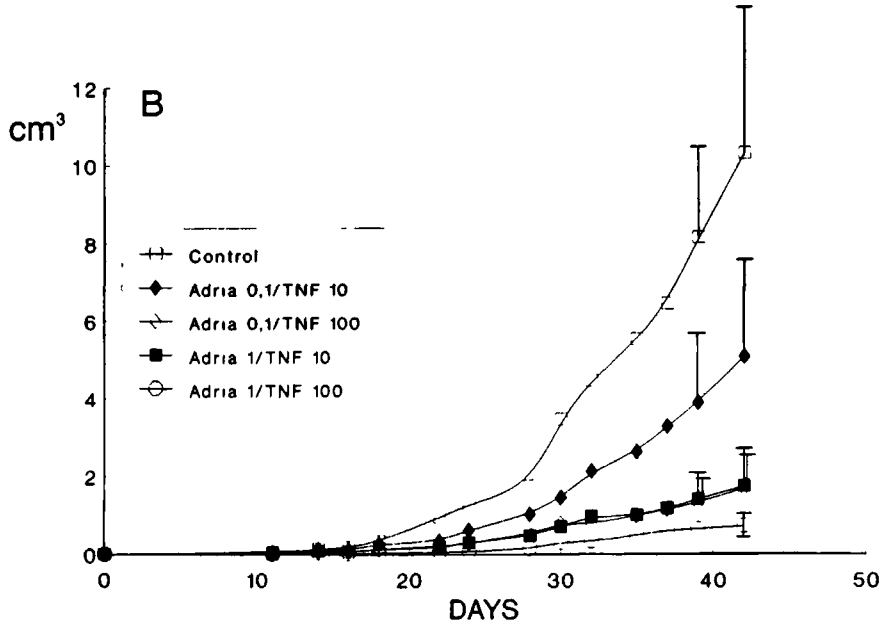


Figure 2. Effects of increase of number of Adriamycin administrations. Treatment started 48 hours after tumor implantation. A: Adriamycin monotherapy administered i.p., for three consecutive days every two weeks. Adriamycin 1 mg/kg has significant antitumor effects. B: Adriamycin and TNF combination therapy. All combinations, except Adriamycin 0.1 mg/kg plus TNF 10 μg, have significant antiproliferative effects. Both combinations including Adriamycin 1 mg/kg have synergistic effects.

Figure 3

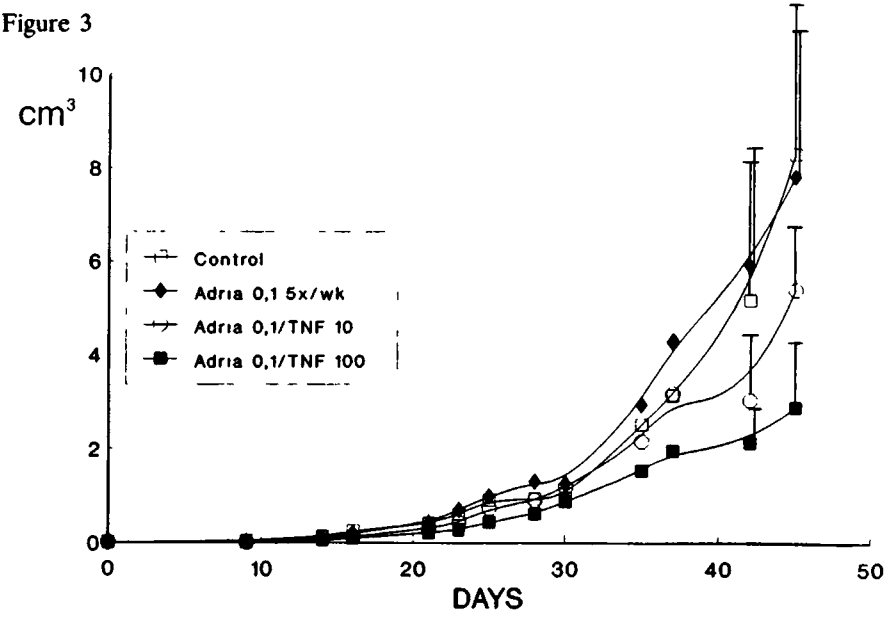


Figure 3. Effect of Adriamycin 0.1 mg/kg, administered i.p., 5 times per week as monotherapy or in combination with s.c., peritumoral TNF.

Figure 4

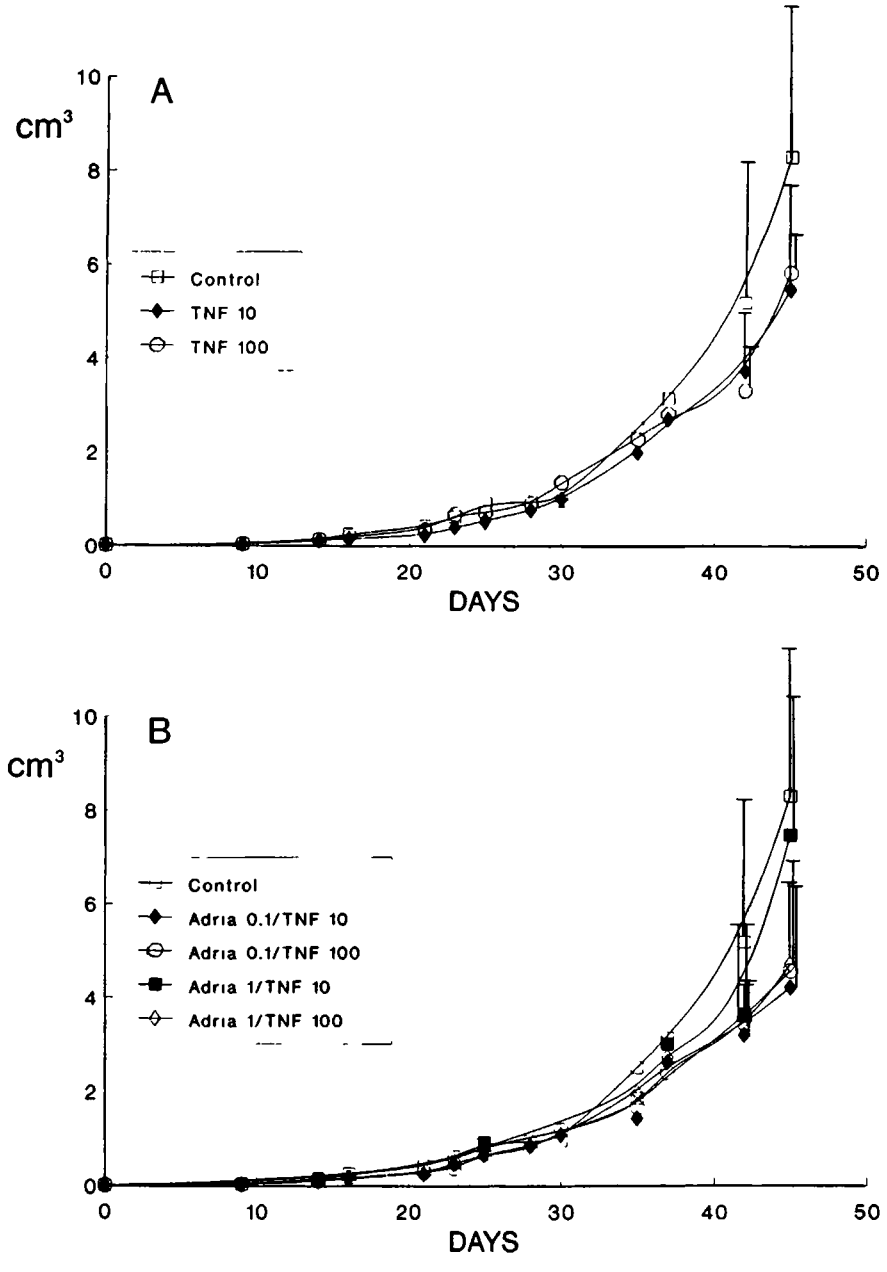


Figure 4. Start of treatment at a tumor volume of 0.2-0.5 cm^3. A: TNF monotherapy administered s.c., peritumorally, 5 times per week. B: Adriamycin 0.1 mg/kg 5 times per week, or 1 mg/kg for three consecutive days every two weeks in combination with TNF.

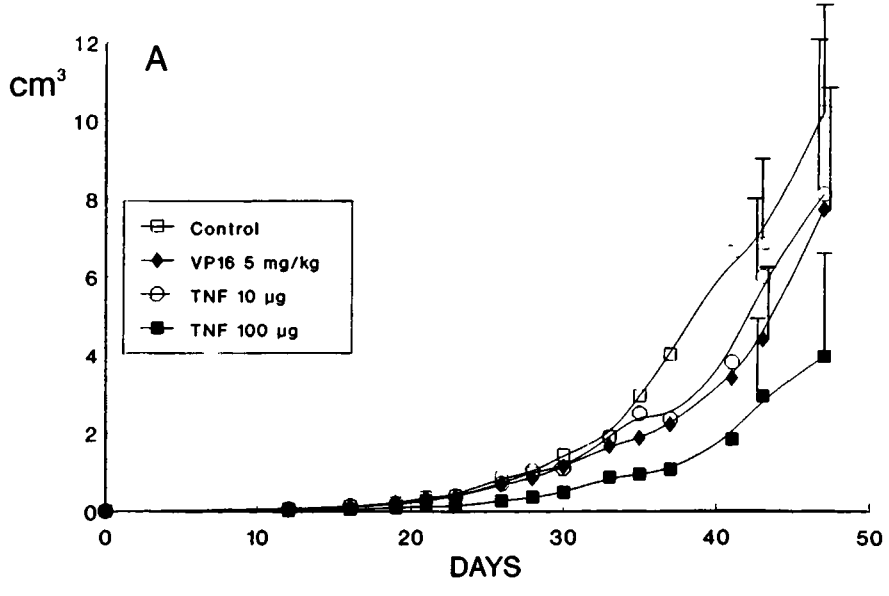
Influence of tumor volume at start of therapy

When treatment started 48 hours after tumor implantation Adriamycin 1 mg/kg for 3 consecutive days every two weeks has synergistic effects in combination with TNF 10 or 100 μg. In order to study, whether this combination treatment or treatment with Adriamycin 0.1 mg/kg 5 times per week, could also provoke regression of larger tumors, we started therapy at a tumor volume of 0.2-0.5 cm^3. TNF monotherapy had no significant effect on tumor growth (fig. 4A), and also Adriamycin monotherapy was ineffective (data not shown). Furthermore, none of the combination regimens produced significant or synergistic effects (fig. 4B).

Antitumor effects of VP16 monotherapy and in combination with TNF

Studies in the murine L1210 leukemia model showed a marked schedule dependency of VP16 antitumor activity. Treatment given on day 1, 5 and 9 was more effective than treatment on day 1 or day 1 and 9 only (Rozencweig *et al.*, 1977). We, therefore, administered in our studies 20, 10, 5 or 1 mg VP16 per kg i.p. at day 2, 6 and 11 after tumor implantation. The highest dose was lethal for 4 out of 6 rats within 5 days after start of therapy, likewise 5 out of 6 rats died within 30 days when treated with VP16 10 mg/kg. All rats treated with 5 or 1 mg VP16/kg stayed alive, however, these lower doses did not produce significant antitumor effects (fig. 5A). In the combined treatment modality, 5 mg VP16 per kg with TNF 100 μg had significant antiproliferative effects, however, substantial augmentation of the therapeutic efficacy was not found (fig. 5B).

Figure 5



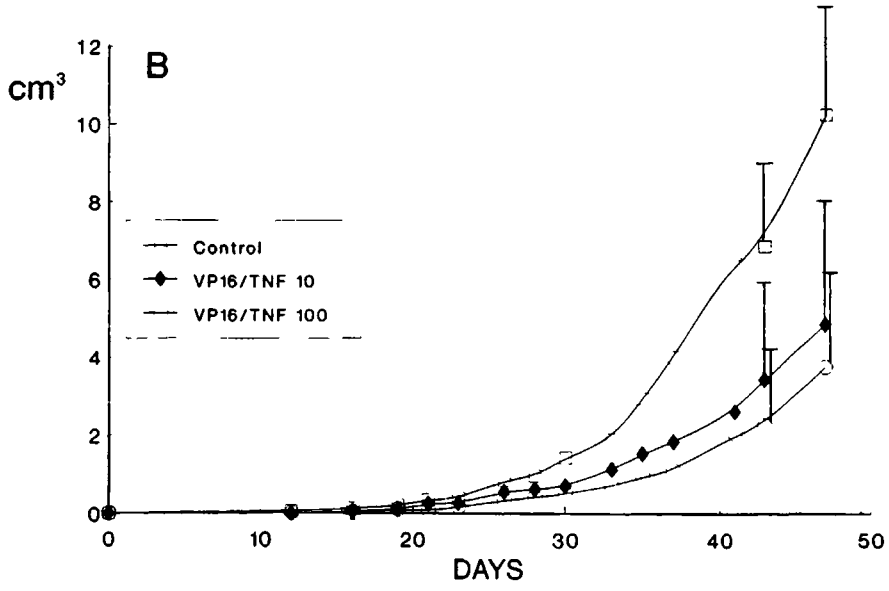


Figure 5. Antitumor effects of combination of VP16 and TNF. A:Monotherapies: VP16 5 mg/kg, administered i.p., on day 2, 6 and 11 after tumor implantation. TNF administered s.c., peritumorally, starting 48 hours after tumor implantation. TNF 100 μg per rat has significant antitumor effects. B: VP16 5 mg/kg and TNF combination therapy. VP16 in combination with TNF 100 μg has significant antiproliferative effects.

DISCUSSION

The treatment of advanced RCC is an intriguing clinical problem. 25 to 30% of patients have metastases at time of diagnosis and more than 80% of patients with disseminated disease will die within three years. Chemotherapy and hormonal therapy give no clearly definable improvement in patient survival (Harris, 1983; Yagoda, 1989). Immunotherapy with e.g. IFN-alpha is associated with an objective response rate of 16% (2% complete and 14% partial responders) in 1,684 treated patients (Horoszewicz and Murphy, 1989).

It was found that TNF has in vitro and in vivo antitumor activity against rodent and human RCC cell lines (Beniers *et al.*, 1989; Kavoussi *et al.*, 1989; van Moorselaar *et al*, 1990; Otto *et al.*, 1990a). In vitro studies show synergistic effects of TNF in combination with other treatment modalities, such as radiotherapy (van Moorselaar *et al*, 1991). The effects of combined treatment of TNF with chemotherapy in experimental cancer models are very promising. Two studies in the nude mouse model showed synergistic effects of VP16 and TNF against human RCC xenografts (Burgers *et al.*, 1989; Donaldson *et al.*, 1990). We have performed in vitro studies in the soft-agar model system to assess the direct growth inhibiting effects of TNF and/or chemotherapy against the rat renal tumor. TNF 10 or 100 ng/dish monotherapy has antitumor effects, whereas VP16 did not significantly

inhibit colony formation. None of the TNF/VP16 combinations induced synergistic effects. In vitro experiments or studies in nude mice are hampered by an absent or abnormal immune system. Despite direct antitumor effects, TNF also has indirect, host response mediated, antitumor effects (Havell *et al.*, 1988). To investigate the full spectrum of TNF mediated effects, we have implanted the tumor in syngeneic Lewis rats. TNF monotherapy has dose-dependent antitumor effects (van Moorselaar *et al.*, 1990). VP16 given singly was lethal in high doses, but lower doses had no significant growth inhibiting effects. Unlike in the xenograft studies, combinations of VP16 and TNF showed no enhanced antitumor activity. These differences in effectivity of VP16 might represent the heterogeneity of RCC, which stresses the need to study different human and animal tumors before one can draw conclusions on clinical applicability of combinations of these drugs.

Intrinsic resistance of RCC to chemotherapeutic agents can be explained by the overproduction of the protein product of the mdr1 gene in a great number of human RCCs (Klein, 1989). Normal rat liver showed expression of the mdr1 gene as described before (Fairchild *et al.*, 1987; Burt and Thorgeirsson, 1988), however, this expression could not be detected in normal rat kidney or the rat renal cell tumor (data not shown). It is therefore likely that other factors than P-glycoprotein are involved in the mechanism of VP16 resistance in the rat renal cell tumor.

In contrast to VP16 and TNF, combination of TNF with Adriamycin shows synergistic inhibition of colony formation by the HM rat RCC cell line. This combination was also effective in vitro against L929, MBT-2 and prostatic cancer cells (Alexander *et al.*, 1987a; Utsugi *et al.*, 1989; Fruehauf *et al.*, 1990). However, 5 lung cancer cell lines appeared to be resistant to TNF/Adriamycin treatment (Giaccone *et al.*, 1990). In the Renca murine RCC model, Adriamycin significantly inhibited tumor growth and increased the effects of immunotherapy with interleukin-2 and lymphokine-activated killer cells (Salup *et al.*, 1987). In contrast to these results, intraperitoneal administration of Adriamycin, starting one day after s.c. tumor implantation every 4 days until day 17, was not effective against another murine renal tumor, the RC tumor, in doses of 6, 3 or 1.5 mg/kg (Vandendris *et al.*, 1983). In our in vivo experiments 1 mg/kg 3 days every 2 weeks showed significant growth inhibition as compared to the control group. Moreover combinations with both doses of TNF induced synergistic effects, but complete growth inhibition was not achieved. Lower doses of Adriamycin or decrease of the number administrations produced less antitumor effects, indicating a dose-dependent tumor growth inhibition (deVere White and Olsson, 1980). When administered repeatedly, Adriamycin appears to accumulate in some tissues, including tumor tissue (Di Marco, 1982). We, therefore, administered Adriamycin in a low dose 5 times per week, but this schedule did not increase the antitumor efficacy of Adriamycin alone or in combination with TNF.

When chemotherapy and immunotherapy are used together, it should be

avoided that the chemotherapeutic agent negates the stimulation of the immune system induced by the immunotherapeutic drugs. Most chemotherapeutic agents are immunosuppressive. Adriamycin, on the contrary, has shown to have little or no immunosuppressive effects (Cohen *et al.*, 1980; Mitchell, 1988). Adriamycin does not interfere with maturation and adherent capacity of human monocytes. However, the drug reduces the expression of Fc-receptors and HLA class II antigens on monocyte-derived macrophages (van Schie *et al*, 1990). It has been shown that TNF can increase HLA class I and II expression of monocytes and tumor cells (Beutler and Cerami, 1988). The antitumor effects and absent or low immunosuppression of Adriamycin, in combination with direct antitumor effects and increase of tumor immunogenicity by TNF, may partially explain the synergistic effects of the combined treatment.

Furthermore, synergistic effects of Adriamycin and TNF might be due to free radical formation and subsequent oxidative tissue damage by both drugs (Bachur *et al.*, 1982; Yamauchi *et al.*, 1990). Moreover, both TNF and topoisomerase-II-targeted drugs can induce DNA breakage, and TNF pretreatment potentiates this breakage and subsequent cell killing by the chemotherapeutic agents (Dealtry *et al.*, 1987; Branellec *et al.*, 1990; Utsugi *et al.*, 1990).

Adriamycin and/or TNF were not effective in inhibiting growth of an established tumor. A similar relation between treatment effectivity and tumor size was found for rat-gamma-IFN and TNF in this rat RCC tumor model (van Moorselaar *et al.*, 1990). Within growing tumors anoxia and/or glucose deprivation can occur, which will lead to an induction of a subset of stress proteins, the glucose-regulated proteins (grps). The lysability of tumor cells by Adriamycin, TNF or cytotoxic T cells decreases due to these grps (Shen *et al.*, 1987; Sugawara *et al.*, 1990). Furthermore, stressed cells can become drug resistant through the depletion of topoisomerase II, which is a consistent characteristic of glucose-regulated stress response and not secondary to the movement of stressed cells into a quiescent state (Shen *et al.*, 1989).

In conclusion, Adriamycin and TNF have in vitro and in vivo synergistic antiproliferative effects against a rat renal cell carcinoma. There is, however, a marked dose and schedule dependency. In contrast to previous studies with RCC xenografts, the combination of VP16 and TNF did not produce synergistic effects. These studies indicate that the combination of chemotherapeutic agents targeted against DNA topoisomerase-II and TNF can have synergistic effects, which might give a lead in the development of new treatment strategies for the clinical management of RCC.

Acknowledgements

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CHAPTER V

SYNERGISTIC ANTITUMOR EFFECTS OF RAT-GAMMA-INTERFERON AND HUMAN TUMOR NECROSIS FACTOR ALPHA AGAINST ANDROGEN-DEPENDENT AND -INDEPENDENT RAT PROSTATIC TUMORS

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ABSTRACT

We have examined the antitumor effects of rat Interferon-gamma (IFN-gamma) and human Tumor Necrosis Factor alpha (TNF) against androgen-dependent and -independent Dunning rat prostatic tumors. In vitro studies, using the double-layer soft-agar assay, showed a very limited antiproliferative activity of the drugs in the dose range tested (1-1000 U IFN-gamma and/or 1-1000 ng TNF per dish). For in vivo studies IFN-gamma and TNF were administered subcutaneously, peritumorally. IFN-gamma was given 3 times per week, 8.000 or 80.000 U per rat, and TNF 5 times per week, 10 or 100 μg per rat. IFN-gamma and TNF monotherapy were not significantly effective in inhibiting tumor growth, except for IFN-gamma against the androgen-independent MatLyLu-tumor. Combinations of IFN-gamma and TNF had synergistic antiproliferative effects against all four tumor lines tested, however, complete growth inhibitions could not be achieved. Survival studies showed significant increase in survival of tumor-bearing rats.

INTRODUCTION

Prostate cancer is the most common malignancy and the second leading cause of cancer related deaths in the USA (1) and due to the aging of the population the incidence and mortality will most likely increase (2). Hormonal therapy is the front-line therapy for patients with advanced or metastatic prostatic cancer (3,4). Approximately 60-70% of these patients initially respond to androgen ablation, but essentially all of these men eventually relapse to a state unresponsive to further antiandrogenic therapy (5). An option for the treatment of relapse and hormone-resistant metastases could be the use of chemotherapy. However, there is still no firm evidence that chemotherapy prolongs survival as compared to palliative therapy. Randomized trials comparing single vs. combination chemotherapy also demonstrated no survival benefit (6,7).

Therefore, new approaches to the treatment of androgen-independent prostatic carcinoma will be required. Immunotherapy with Biological Response Modifiers, like Interferons (IFNs) and Tumor Necrosis Factor Alpha (TNF), has been adopted effectively in some types of malignancies (8,9). IFN-gamma is a product of activated T-lymphocytes with potent antiviral, antiproliferative and immunomodulatory properties such as the activation of natural killer cells and macrophages, the increase of expression of tumor associated antigens and the modulation of MHC class I and II antigens (10). Interferons showed antiproliferative effects against prostatic cancer cell lines in vitro (11).

In 1975 Carswell et al. noted that post-endotoxin serum derived from mice treated with Bacillus Calmette-Guerin (BCG) was able to induce haemorrhagic necrosis of experimental tumors. The substance responsible for these effects was designated TNF (12). It is synthesized by various activated phagocytic and non-phagocytic cells, including macrophages/monocytes. TNF has pleiotropic effects, for example, it causes cytostasis or cytolysis of a variety of animal and human tumors (13). When exposed to TNF endothelial cells produce a procoagulant factor, and show decreased expression of thrombomodulin, these effects favor the accretion of thrombus on endothelial surfaces, which may be related to the haemorrhagic necrosis of tumors (14). TNF showed antitumor effects against the LNCaP, DU145 and PC3 prostatic xenografts in vitro and in vivo (15).

We have examined the antitumor effects of IFN-gamma and TNF in the Dunning rat prostatic tumor model. This model consists of a number of sublines that differ widely in their histology, growth rate, androgen sensitivity and metastatic abilities. An additional advantage of this system is that from a number of in vivo tumor lines, in vitro cell lines have been derived that retain their phenotypic characteristics (16,17). All sublines are transplantable in immunocompetent rats and it is, therefore, possible to study the full spectrum of cytokine mediated effects. We show here that IFN-gamma and TNF can have synergistic antitumor effects against

rodent androgen-dependent and -independent prostatic tumors. Although it is very difficult to extrapolate animal data to the human situation, these studies may give a lead to the development of future treatment strategies.

MATERIAL AND METHODS

Animals

Adult male F_1 hybrid rats (F344CopF1/OLA/HSD, Copenhagen male and Fisher female) were obtained from Harlan OLAC Limited, Bicester, England and kept 3 rats per cage with a regimen of 12 hours light per day. The animals were allowed access to RMH-TM food (Hope Farms BV., Woerden, The Netherlands) and water ad libitum.

Tumor Lines

Four well-characterized sublines of the Dunning tumor were kindly provided by Dr. J.T. Isaacs (Johns Hopkins Oncology Center, Baltimore, USA). The characteristics of the H-, PIF-1-, PAT-2- and MatLyLu-sublines are summarized in Table I.

In vitro cell lines were maintained in RPMI 1640 culture medium supplemented with 10% fetal calf serum, 2mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (GIBCO Ltd, Paisley, Scotland), 250 nM dexamethasone and 20 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (Sigma, St. Louis, MO, USA).

For in vivo studies tumors were excised from tumor-bearing animals, and after removal of normal and necrotic tissue, the tumor tissue was cut into pieces of 20 mg and implanted into the right flank of recipient animals as described before (18).

In vitro testing with the double layer soft-agar assay.

Single cell suspensions were obtained after quick trypsinization of subconfluent monolayer cultures. Cell density and viability were determined by adding 15 μl trypan blue solution (25 mg in 5 ml 3% acetic acid) to 15 μl cell suspension and simultaneously counting colored and non colored cells using a Bürker Türk hemocytometer. Suspensions with a viability of 95% or more were used for in vitro testing. For the detection of the in vitro growth potential a modified double layer soft agar culture method as originally described by Salmon and Hamburger was used (19). An underlayer of one ml 0.5% agar in double enriched (DE) McCoy's 5A medium was plated in 35 mm dishes and allowed to gel at room temperature. Tumor cell suspensions were plated at their optimal cell concentrations, i.e. concentration of cells that will give rise to formation of 200-500

colonies after 14 days culture, in the upper layer consisting of DE-CMRL 1066 medium with 0.3% agar. For the MatLyLu cell line the optimal starting concentration is 750 cells per dish (17). In a pilot study we plated a range of 500 to 100,000 cells of the PIF-1 and PAT-2 cell lines. The optimal starting concentrations appeared to be 2,000 and 35,000 cells for the PAT-2 and the PIF-1 cell line respectively. Cells were cultured immediately after preparation of the single cell suspension. Dishes were incubated at 37°C in 6% CO_2 in air with 100% humidity. Growth potential was quantified using an Omnicon Fas II automated colony counter (Milton Roy Inc., Rochester, New York) (20). Results are presented as the growth potential of cells relative to the untreated control cells.

Drugs

Recombinant human TNF-alpha was kindly provided by Boehringer Ingelheim (Alkmaar, The Netherlands). The specific activity of TNF determined in the presence of Actinomycin D was 6 x 10^7 Units/mg protein as determined in the L-929 cytotoxicity assay. The purity was >99% as determined by SDS polyacrylamide gel electrophoresis and it contained less than 1.0 ng endotoxin per mg protein based on the limulus amebocyte assay. The original TNF solution was diluted with RPMI 1640 culture medium to a final concentration of 20, or 200 μg per ml, divided in aliquots and stored at -20°C.

Recombinant rat IFN-gamma was produced by a Chinese hamster ovary cell line transformed with a plasmid carrying the chromosomal gene for rat IFN-gamma and purified as described previously (21). The cytopathic effect reduction assay was used for estimating IFN bioactivity. Ratec cells (22) (a continuous rat embryonic cell line) were incubated with IFN-gamma for 18 hours before they were challenged with vesicular stomatitis virus to determine the cytopathic effect. Because no internationally accepted reference standard for rat interferon is available, all titers were determined by comparison with a laboratory standard preparation of IFN-gamma (1 mg pure rat IFN-gamma corresponds with 7 x 10^6 units). After purification by monoclonal antibody affinity chromatography IFN-gamma was freeze-dried for longterm storage. It was reconstituted with Milli-Q to a final concentration of 16,000 or 160,000 Units per ml, divided in aliquots and stored at -80°C until use.

Treatments and monitoring

In vitro drug tests were performed by continuous exposure of the drugs to the tumor cells. Stock solutions of IFN-gamma and TNF were diluted with DE-CMRL 1066 medium to concentrations of 50, 500 or 5,000 U or ng/ml. The BRMs were layered over the upper layer in a volume of 200 μl, 1 hour after gelling of the upper layer. Dishes in the control group received 200 μl DE-CMRL 1066 medium only. As a cytotoxic control we used 0.37 mM $HgCl_2$. In vitro tests were performed in triplicate and repeated at least two times.

Table I. Biological characteristics of the Dunning R3327 rat prostatic cancer sublines used in this study

Tumor	Histology	In vitro cell line	Growth rate in vivo[a]	Androgen Sensitivity	Metastatic Potential[b]
H	well differentiated	no	22 ± 5 days	yes	< 5%
PIF-1	moderately differentiated	yes	3.7 ± 1.1 days	no	< 5%
PAT-2	anaplastic	yes	1.8 ± 0.2 days	no	> 90%
MatLyLu	anaplastic	yes	1.5 ± 0.1 days	no	> 90%

[a] ref. 17 and own observations

[b] number of s.c. inoculated rats that develop metastases to lymph nodes and lungs

For in vivo studies IFN-gamma and TNF were administered s.c., peritumorally, 3 and 5 times per week respectively, in a 0.5 ml injection volume. Phosphate buffered saline (PBS) was used for the control group. Treatment started 2 days after tumor implantation and was continued until the tumors in the control group reached a volume of 10 cm^3. However, in survival experiments treatment was continued until death of the animals.

For the H tumor a different treatment protocol was used. Treatment started when the tumor became palpable, this is 8 weeks after implantation. IFN-gamma was administered two times/week, from day 56 until day 112 after tumor implantation. TNF was given 4 times/week, from day 56 until day 205. All experiments, except for the H tumor, were carried out in duplicate.

Tumor growth was followed by measurements of tumor size according to the method of Janik et al. (23) by the same measurer throughout each experiment. Once weekly for the H tumor, and three times per week for the other three tumors, length (l), width (w) and height (h) were measured using a slide caliper. No correction was made for skin thickness. Tumor volume (V) was calculated as

$$V = 0.5236 \times l \times w \times h.$$

Statistical analysis

Statistical analysis of the in vitro data was done by analysis of variance on the logarithms of the colony number at day 14 after seeding. Differences between mean colony numbers were tested for significance using the Tukey studentized range test at an experimental 0.05 error rate (24).

For each rat, the tumor volume was estimated on the last treatment day by loglinear regression on the tumor volumes throughout the treatment period. Analysis of variance was used to compare the final volumes of the treatment versus the control groups and to test for additive effects in the combination treatments. To test the significance of differences between groups the Tukey studentized range test was used at an experimental 0.05 error rate (24). 6 animals were included in each group.

The effect of treatment A is defined as $E_A = V_{control}/V_A$ and the effect of treatment B as $E_B = V_{control}/V_B$, where V_A, V_B, and $V_{control}$ are the estimates for the tumor volumes of treatment A, B or control group respectively. Additivity is defined (25) as $E_{A+B} = E_A \times E_B$ (i.e. $\log E_{A+B} = \log E_A + \log E_B$). A combination of two treatments is synergistic when the effect of the combination (E_{A+B}) is greater than the product of the effects of the separate treatments.

Effect of IFN-gamma and TNF in vitro

35,000, 2,000 and 750 cells per dish, for the PIF-1, PAT-2 and MatLyLu subline, respectively, were plated in the upper layer of the double-layer soft-agar culture system. Table II demonstrates the effects of IFN-gamma and TNF as single exposure. Treatment started on the day of cell seeding. Combined results of three separate experiments are expressed as relative growth potential, i.e. number of colonies in treated dishes divided by number of colonies in control dishes after 14 days of culture. Colony formation, however, was monitored every 3 days after seeding and also in the initial colony counting the same relative differences were found in treated and untreated groups. The results, therefore, cannot be attributed to catch-up growth after a single administration of BRMs.

IFN-gamma monotherapy has dose-dependent antiproliferative effects against the PIF-1 and the PAT-2 cell lines. IFN-gamma showed significant growth inhibiting effects in 3 out of 4 dose levels for the PIF-1 cell line and for the highest dose against the PAT-2 cell line. Increase of IFN-dose from 1 to 1000 U per dish, however, did not increase the effect of IFN-gamma on the MatLyLu cell line. TNF monotherapy had no significant effects on colony formation in all three cell lines.

To determine whether combinations of cytokines might enhance the direct antiproliferative effects we combined IFN-gamma in a dose range from 1 to 100 U per dish with TNF 1 to 100 ng/dish. Both drugs were added at the same time. The combination of 100 U IFN-gamma and 100 ng TNF per dish gave rise to significant antiproliferative effects in all three cell lines. According to the definition of synergism, whereby the effect of the combination must be greater than the product of the two separate treatments, only the combination of 10 U IFN-gamma and 100 μg TNF per dish has synergistic antiproliferative effects against the MatLyLu cell line. All other combinations did not produce synergistic effects.

Effect of IFN-gamma and TNF against the androgen-dependent H tumor in vivo.

In previous studies with a rat renal cell carcinoma model system we have evaluated the influence of administration route on antitumor effects of the BRMs (18). Comparison of the intravenous (i.v.), intraperitoneal (i.p.) and subcutaneous (s.c.) route showed that i.p. and i.v. injected BRMs were less effective than s.c. injections. Therefore, in the in vivo experiments described here we administered the BRMs s.c., peritumorally.

Tumor pieces of 20 mg of the H tumor were implanted s.c. and treatment was started, in contrast to the other three tumors, 8 weeks after tumor implantation. IFN-gamma was given 2 times and TNF 4 times/week. As shown in fig 1A IFN-gamma monotherapy with 8,000 or 80,000 U per rat has no significant antitumor effects. TNF monotherapy with 10 or 100 μg/rat also could not significantly inhibit

Table II. Effects of IFN-gamma and TNF in vitro

treatment		PIF-1 relative growth potential	PAT-2 relative growth potential	MatLyLu relative growth potential
IFN[a]	TNF[b]			
0	0	1.00 ± 0.10	1.00 ± 0.04	1.00 ± 0.05
1000	0	0.43 ± 0.09[c]	0.75 ± 0.08[c]	0.82 ± 0.07
100	0	0.67 ± 0.11[c]	0.80 ± 0.06	0.76 ± 0.05
10	0	0.77 ± 0.16	0.95 ± 0.06	0.73 ± 0.07
1	0	0.75 ± 0.16[c]	0.96 ± 0.05	0.71 ± 0.07
0	1000	0.92 ± 0.18	0.73 ± 0.03	0.71 ± 0.03
0	100	0.95 ± 0.14	0.83 ± 0.07	1.01 ± 0.08
0	10	1.03 ± 0.13	0.91 ± 0.09	0.84 ± 0.11
0	1	1.06 ± 0.14	0.90 ± 0.05	1.03 ± 0.09
100	100	0.71 ± 0.11[c]	0.67 ± 0.06[c]	0.53 ± 0.06[c]
100	10	0.74 ± 0.13	0.64 ± 0.06[c]	0.72 ± 0.06
100	1	0.73 ± 0.10	0.80 ± 0.05	0.79 ± 0.08
10	100	0.93 ± 0.13	0.80 ± 0.05	0.55 ± 0.13[c,d]
10	10	0.88 ± 0.14	0.79 ± 0.04	0.65 ± 0.07[c]
10	1	0.79 ± 0.14	0.81 ± 0.04	0.82 ± 0.11
1	100	0.97 ± 0.16	0.85 ± 0.06	0.70 ± 0.10
1	10	0.85 ± 0.15	0.89 ± 0.04	0.95 ± 0.12
1	1	0.86 ± 0.13	0.96 ± 0.05	0.82 ± 0.06

[a] IFN-gamma dose in U per dish
[b] TNF dose in ng per dish
[c] significantly different colony numbers compared to untreated control
[d] synergistic IFN-gamma and TNF combination treatment

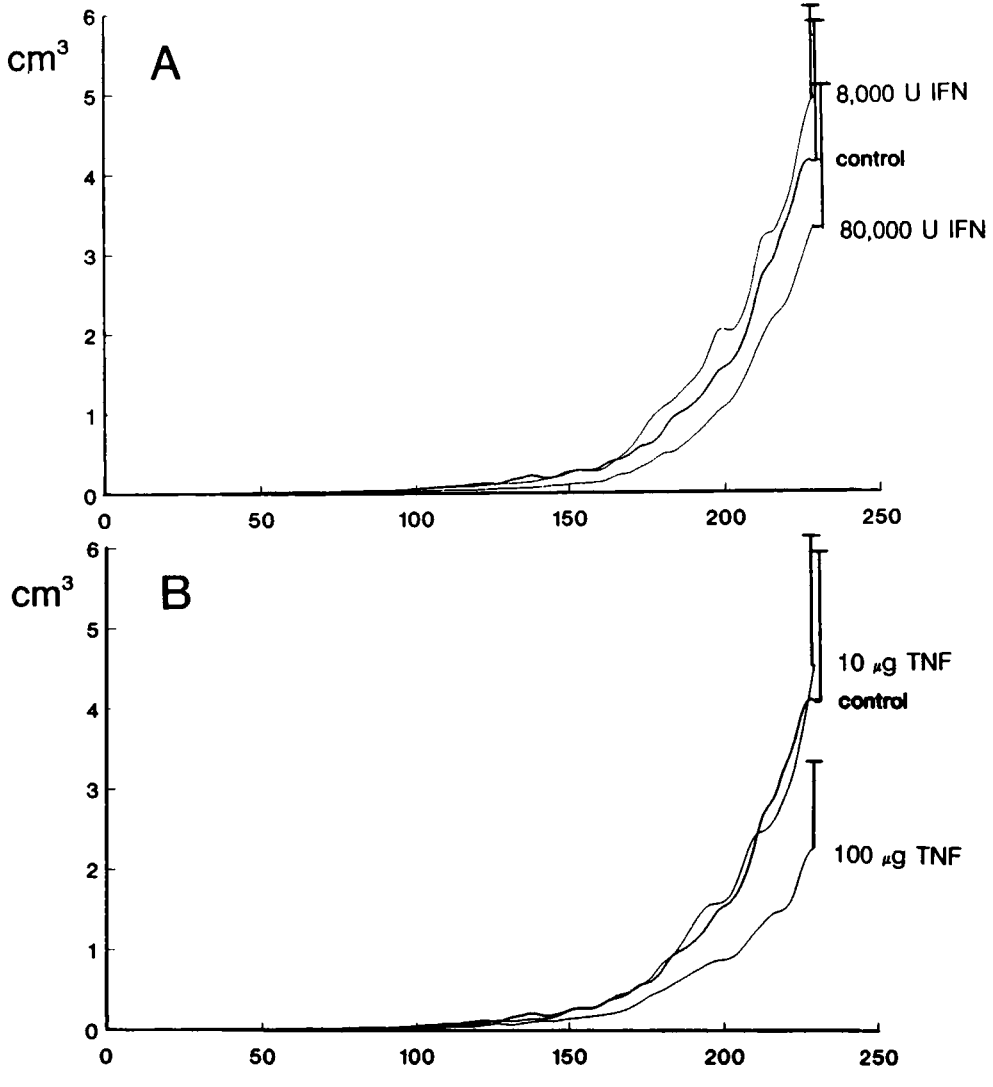
Table II. The PIF-1, PAT-2 and MatLyLu cell lines were plated in the upper layer of the double layer soft-agar culture system. IFN-gamma and TNF were added, in a dose range of 1 to 1000 U or ng per dish respectively, on the day of cell seeding. Combined results of three separate experiments are expressed as relative growth potential, i.e. number of colonies in treated dishes divided by number of colonies in control dishes after 14 days of culture.

tumor growth of the H tumor (fig 1B).

Reasoning that combination of IFN-gamma and TNF can have synergistic effects, we combined 8,000 or 80,000 U IFN-gamma with 10 or 100 μg TNF (fig 1C). The combinations of 8,000 U IFN-gamma with 10 μg or 100 μg TNF did not significantly reduce tumor growth. Substantial augmentation of the therapeutic efficacy resulted from combined treatment with the highest dose of IFN-gamma and TNF. Tumor regression, however, could not be achieved.

The highest doses of BRMs produce the best effect, suggesting a dose-dependent response relationship. According to the definition of synergism only the combination of 80.000 U IFN-gamma and 100 μg TNF has synergistic effects.

Figure 1



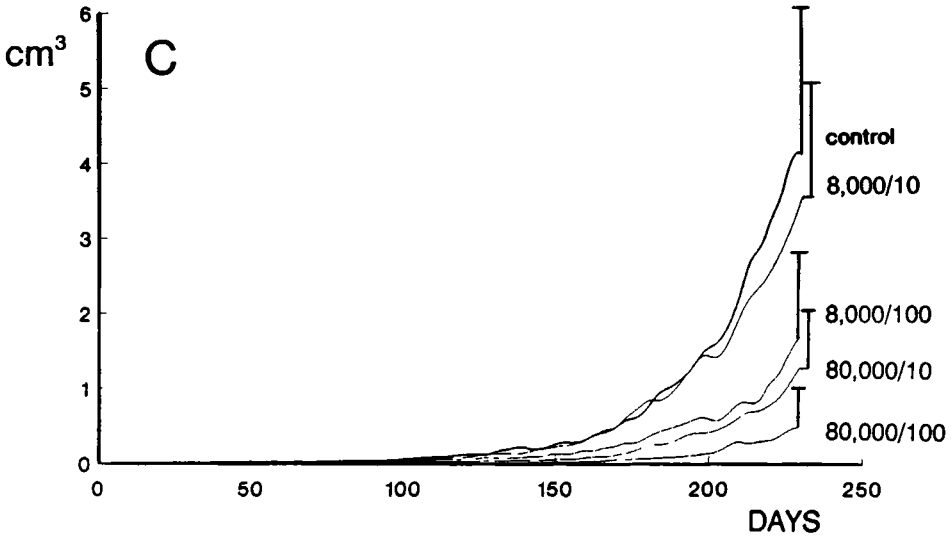


Figure 1. Antitumor effects of IFN-gamma and TNF against the androgen-dependent H-tumor. Treatment started 57 days after tumor implantation. A: IFN-gamma monotherapy, administered s.c. peritumorally, 8,000 or 80,000 U per rat, 2 times per week. B: TNF monotherapy, administered s.c. peritumorally, 10 or 100 μg per rat, 4 times per week. C: IFN-gamma and TNF combination therapy, the combinations of 80,000 U IFN-gamma plus 10 or 100 μg TNF per rat have statistically significant antitumor effects.

Effects of IFN-gamma and TNF against the androgen-independent PIF-1, PAT-2 and MatLyLu tumors in vivo.

Having found a drug combination that could synergistically inhibit the slow-growing, androgen-dependent H-tumor, we sought to gain insight into the antitumor effects against androgen-independent tumors.

Tumor pieces (20 mg) of the PIF-1, PAT-2 and MatLyLu tumors were implanted s.c. IFN-gamma was administered 3 times and TNF 5 times/week. IFN-gamma or TNF monotherapy could not significantly inhibit tumor growth of the moderately differentiated PIF-1 tumor (fig. 2). IFN-gamma and TNF in combination, however, mediated a synergistic reduction in tumor growth.

TNF monotherapy was not effective against the two rapidly growing anaplastic tumors, MatLyLu and PAT-2 (figs. 3 and 4). IFN-gamma monotherapy, however, was very effective against the MatLyLu tumor. The substantial benefit of IFN-gamma alone made it difficult to reach synergism, but combination therapy was significantly more effective than TNF or IFN-gamma alone. In the PAT-2 tumor combination treatment was also more effective than each of the monotherapies and produced synergistic effects.

In experiments with the androgen-independently growing tumors it appeared that, after termination of therapy, after a 10-day lag phase the tumors regained their initial growth potential (same volume-doubling time, data not shown).

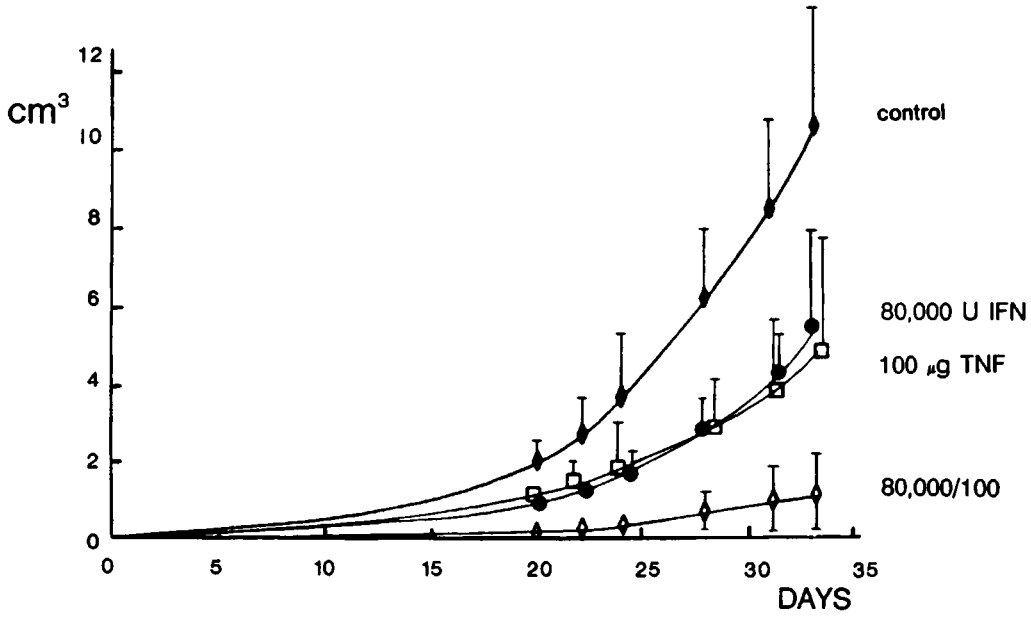


Figure 2. Effects of 80,000 U IFN-gamma and/or 100 µg TNF per rat, 3 or 5 times per week respectively, against the androgen-independently growing PIF-1 tumor. Combination treatment has statistically significant antitumor effects.

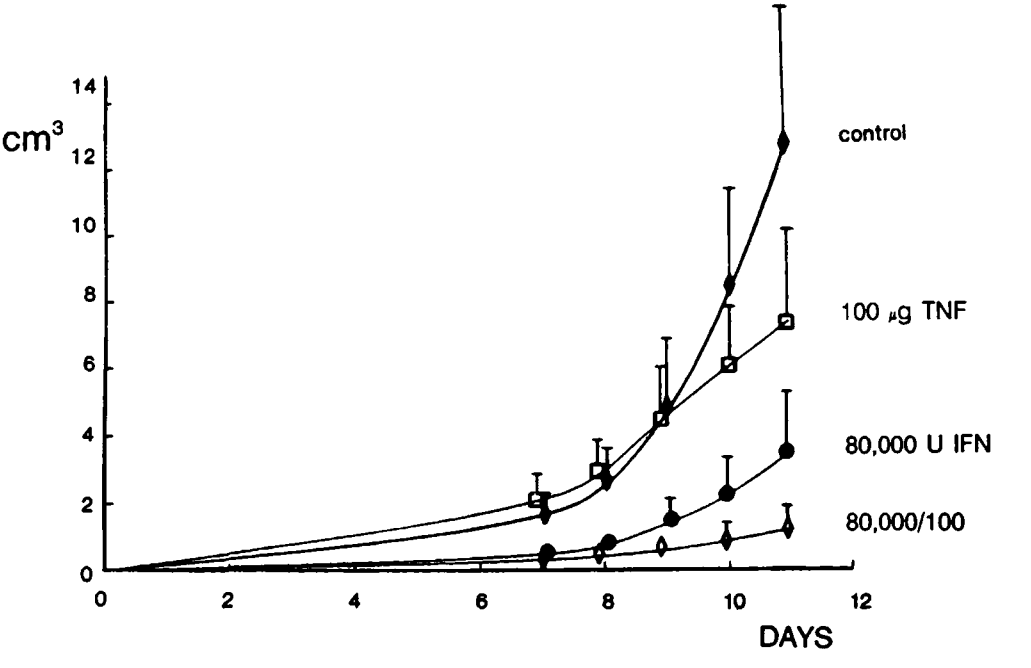


Figure 3. Effects of 80,000 U IFN-gamma and/or 100 µg TNF per rat, 3 or 5 times per week respectively, against the androgen-independently growing MatLyLu tumor. IFN-gamma monotherapy and combination treatment have statistically significant antitumor effects.

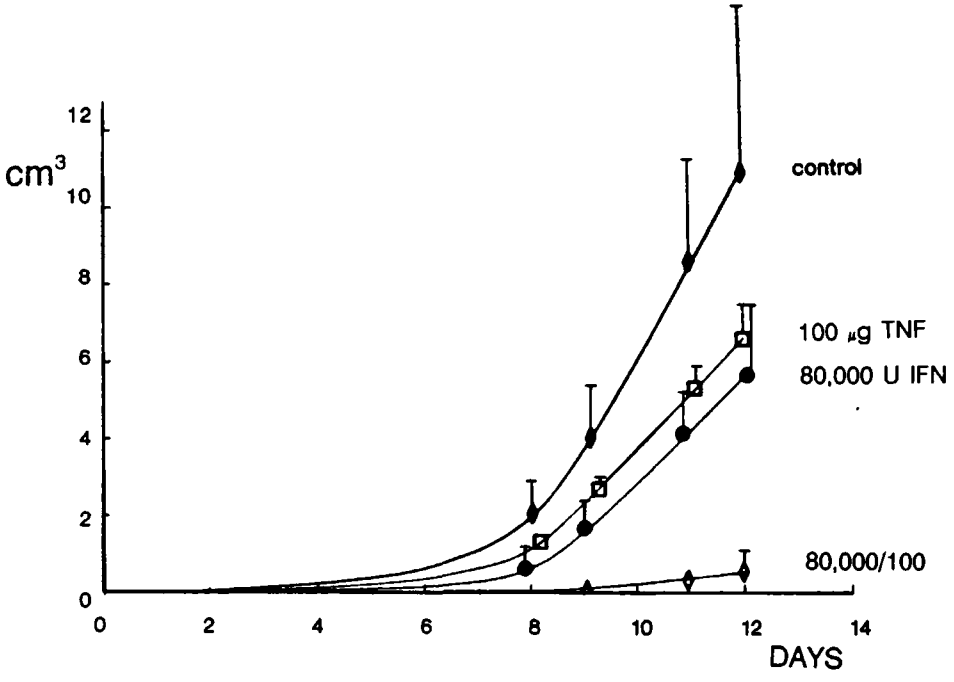


Figure 4. Effects of 80,000 U IFN-gamma and/or 100 µg TNF per rat, 3 or 5 times per week respectively, against the androgen-independently growing PAT-2 tumor. Combination treatment has statistically significant antitumor effects.

Survival of rats with MAtLyLu-tumors under continuous treatment of IFN-gamma and TNF.

To investigate whether IFN-gamma and TNF combination therapy influenced survival of rats we treated MatLyLu-bearing rats with IFN-gamma 3 times and TNF 5 times/week until death. As shown in fig. 5 all rats finally died, but the treated rats had a survival benefit of 11 days when compared to the untreated controls (40 days vs. 29 days). This difference is significant with the Wilcoxon test ($p=0.005$).

DISCUSSION

Androgen ablation is the front line palliative treatment for advanced or metastatic prostatic carcinoma. From 60 to 70% of the patients will respond to hormonal therapy. However, following this initial positive response a nearly universal relapse will follow to an androgen-insensitive state. The median survival of these patients is between 4 and 8 months. To date chemotherapy showed no substantial improvement in the survival of patients with hormone-escaped metastatic prostatic cancer. Clearly novel therapeutic interventions must be developed. Medenica et al.

Figure 5

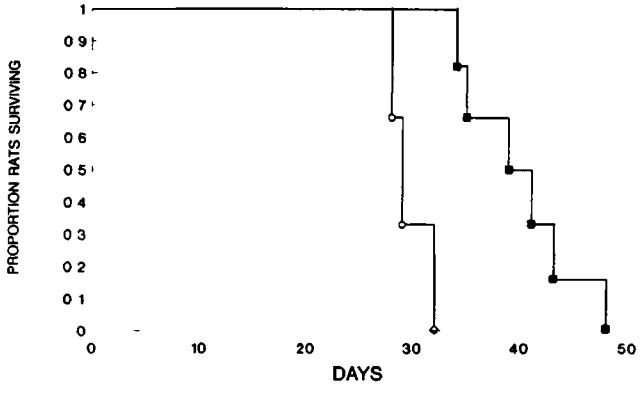


Figure 5. Survival of MatLyLu-tumor bearing rats. 0–0 untreated controls, ▮–▮ rats treated with 80,000 U IFN-gamma, 3 times per week and 100 μg TNF, 5 times per week. The difference is statistically significant as evaluated by the Wilcoxon test.

treated 14 patients with IFN-alpha and observed 5 complete responses and 6 partial responses (26). Gutterman and Quesada also found encouraging results with IFN-alpha (27). They found 1 complete response and 3 partial responses among 10 treated patients. Other authors (28,29), however, could not confirm objective clinical responses with IFN-alpha or -beta. The number of patients treated with these two types of IFN is too small to draw firm conclusions about clinical activity of the drugs. We have investigated the antiproliferative efficacy of immunotherapy with IFN-gamma and TNF, in vitro and in vivo in the Dunning rat prostatic tumor model system. The syngeneic Dunning R3327 system of serially transplantable rat prostatic cancers mimics many of the most important characteristics of the human disease, and serves, therefore, as an appropriate model system.

In vitro tests were performed to investigate the extent to which the antitumor effects of the BRMs in vivo can be contributed to *direct* growth inhibiting effects. For these studies we used the soft agar assay of Hamburger and coworkers, which has been suggested as a relevant system for preclinical evaluation of potential new agents. We have shown previously that IFNs and TNF can inhibit colony formation of renal cell carcinoma cells in this assay (30). The in vitro studies presented here revealed a very limited activity of IFN-gamma and TNF alone or in

combinations, indicating that both cytokines have a very limited direct cytostatic or cytotoxic effect on prostatic cancer cell lines in the dose range tested.

Horoszewicz et al. could not demonstrate antiproliferative effects of highly purified human beta-interferon (HuIFN-beta) against the hormone-sensitive LNCaP prostatic cancer cell line in vitro (31). In contrast to these results, Sica et al. showed that HuIFN-beta and recombinant human IFN-alpha (rHuIFN-alpha) had growth-inhibiting effects against the hormone-insensitive PC3 and DU145 prostatic cancer cell lines in vitro (11). Sherwood et al. reported cytotoxicity of TNF towards all three cell lines in vitro (15); Fruehauf et al., however, found that the PC-3 cell line was almost completely resistant to the action of TNF whereas the LNCaP cells were sensitive targets for TNF (32).

IFN-gamma and TNF can have direct effects but, more importantly, also indirect, immune system-mediated effects. There is little opportunity to determine this indirect drug activity using the basic clonogenic assay. Inclusion of macrophages in the underlayer could be a possibility to optimize the assay, since addition of macrophages inhibited colony formation more than IFN-gamma treatment alone, due to the secretion of soluble substances by these macrophages, which consist in part of TNF (33).

In vivo studies showed that IFN-gamma or TNF monotherapies, except IFN-gamma against the MatLyLu tumor, were not significantly effective in inhibiting rat prostatic tumor growth. Shaw et al. also used the MatLyLu tumor (34). They reported that a single administration of TNF 30 μg i.p. had no antitumor effect, whereas intratumoral (i.t.) injection significantly reduced tumor growth. Administration of TNF i.v. to nude mice bearing s.c. or intraabdominal PC-3 tumors resulted in a significant growth reduction (15). These results indicate that the optimal route of administration for cytokines has not been established yet.

Since both IFN-gamma and TNF monotherapy had limited activity we combined these drugs. With the H tumor we performed dose-finding studies and found out that the combination of 80,000 U IFN-gamma and 100 μg TNF had synergistic antiproliferative effects. This combination was also tested against more rapidly growing, androgen-independent tumors. Synergistic effects were apparent in the PIF-1 and PAT-2 tumors. Against the MatLyLu tumor the combination was more effective than the monotherapies in the dose range tested. IFN-gamma monotherapy was already too active to show synergism in the combination.

In survival studies we showed that combined treatment with IFN-gamma and TNF significantly prolonged survival of MatLyLu-bearing animals compared to untreated controls. Shaw et al. also showed a survival benefit for i.t. treated rats with TNF alone (24 days compared to 31 days) (34).

The involution following androgen-ablation is an actively initiated process, induced by a decrease of prostatic androgens beyond a critical point (35). This active energy-dependent process which involves a cascade of biochemical changes,

like fragmentation of the genomic DNA into the nucleosomal oligomers (multiples of a 180-nucleotide base pair subunit) is referred to as programmed cell death (36). Recently it has been shown that androgen dependent prostatic cells retain the ability to activate programmed cell death in response to androgen ablation (37). In androgen-independent prostatic cancer cells, however, there is a defect such that the programmed cell death is no longer activated by androgen removal. TNF exerts its anticellular effect in a similar way, by degradation of DNA into nucleosome fragments, and this effect can be enhanced by IFN-gamma (38,39). This may in fact explain the synergistic effects of combinations of these cytokines. Immunotherapy with biological response modifiers could be a method to activate the programmed cell death in androgen-independent cancer cells.

The experimental data presented here indicate that cytokines can have antiproliferative effects against hormone-independent cancer cells. This may open a way to a new therapeutic modality in the treatment prostatic cancer.

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CHAPTER VI

DIFFERENTIAL ANTIPROLIFERATIVE ACTIVITIES OF ALPHA- AND GAMMA-INTERFERON AND TUMOR NECROSIS FACTOR ALONE OR IN COMBINATIONS AGAINST TWO PROSTATE CANCER XENOGRAFTS TRANSPLANTED IN NUDE MICE

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ABSTRACT

We have investigated the antiproliferative effects of recombinant human alpha- and gamma-Interferon (IFN) and recombinant human Tumor Necrosis Factor alpha (TNF) against the hormone-independently growing PC3 and DU145 prostatic tumor lines. Subcutaneous, peritumoral administration of the drugs was started 24 hours after subcutaneous implantation of 1-2 mm^3 tumor pieces. IFN was given three times per week and TNF five times per week. IFN-alpha (dose-range 0.5-5 ng/gram bodyweight) had significant growth-inhibiting effects against the PC3 tumor, but showed no significant antitumor effects against the DU145 tumor. IFN-gamma monotherapy (dose-range 8-80 ng/g body weight) was less effective than IFN-alpha. 500 ng/gram TNF produced growth inhibition of both tumors, whereas the lower dose (50 ng/g) was only effective against the PC3 tumor. IFN-alpha and -gamma combination treatment had significant antiproliferative effects against the PC3 tumor, but not against the DU145 tumor. Combinations of IFN-alpha and TNF were very effective against both xenografts, some combinations resulted in complete growth inhibition. IFN-gamma and TNF combinations also showed significant antitumor effects against both tumor lines. We therefore conclude that cytokine combination treatment may provide a new approach in the treatment of hormone-escaped prostatic tumors.

INTRODUCTION

Prostatic carcinoma is the most common tumor occurring among men (1). The tumor is found most frequently in men of 50 years or older, with the incidence rising with each decade. Furthermore, the incidence and mortality will most likely increase due to the aging of the population (2).

The disease is frequently at an advanced stage at the time of presentation: more than 60% (range 21-80%) of the patients have disease extended beyond the prostate (3). The management of both regional and metastatic prostatic cancer remains a major clinical problem. Since Huggins and Hodges' report on the androgen dependency of prostatic carcinoma in 1941, androgen ablation has been the front-line therapy for prostatic cancer (4). Approximately 60-70% of the patients will initially respond to androgen ablation (5). Unfortunately, however, essentially all of these patients will relapse to a state unresponsive to further antiandrogenic therapy. To date, chemotherapy has not prolonged survival of patients with metastatic prostatic carcinoma (6,7). Therefore, new approaches to the treatment of, especially the androgen-independently growing, prostatic tumors must be developed.

Next to surgery, chemotherapy and radiation therapy, immunotherapy is the fourth cancer treatment modality. Cytokines, like Interferons (IFN), show promising results in experimental model systems as well as in phase I and II clinical studies (8,9). Three main groups of IFN can be defined, IFN-alpha, IFN-beta and IFN-gamma (10). Alpha-IFNs are a group of closely related proteins with approximately 75% homology in their amino acid sequences. They are produced mainly by leukocytes upon exposure of viruses, bacteria, and double-stranded RNA, and share the same cell membrane receptor with IFN-beta. IFN-gamma is the product of activated T-lymphocytes, and is encoded by a unique gene which has little or no homology to the other two IFN-types (11). IFNs have direct antiproliferative effects against tumor cells and indirect immune modulating effects, e.g. they can induce or enhance the expression of cell surface antigens of the major histocompatibility complex (MHC) and stimulate cytotoxic T-cells, NK-cells and macrophages. In vitro studies with prostatic cancer cell lines showed direct antiproliferative effects of IFN-alpha and -beta (12,13). Some clinical trials have already been conducted with different IFNs. The number of patients treated with IFN, however, is too small to draw firm conclusions about clinical activity of these drugs in prostate cancer (8).

TNF is the cytokine in serum of mice treated sequentially with BCG and endotoxin that promotes tumor lysis (14). It is cytotoxic/cytostatic to some tumor lines in vitro (approximately 30% of the tested lines) and it can cause hemorrhagic necrosis in transplanted tumors in vivo (14,15). TNF is produced by activated macrophages and showed antiproliferative effects against prostatic tumor lines in vitro and in vivo (16,17).

As shown for other solid tumors, combinations of alpha- and gamma-IFN

and TNF can result in the enhancement of their respective antitumor effects in vitro and in vivo (18,19,20). In the studies presented here we tested the antiproliferative effects of recombinant human IFN-alpha and -gamma and TNF alone or in combinations against two androgen-independently growing prostatic xenografts transplanted in nude mice.

MATERIAL AND METHODS

Animals

Six weeks old male Balb/C nu/nu mice were obtained from Bornholt Gård, Rije, Denmark. Groups of five mice were kept in PAG type 2 cages covered with an iso cap (Iffa Credo, France) for sterile conditions and with a regimen of 12 hours light per day. Sterilized sawdust (Iffa Credo Broekman BV, The Netherlands) was used as bedding material inside the cages. Mice were allowed access to gamma irradiated (0.9 MR) SRM-A MM food (Hope Farms, Woerden, The Netherlands) and water ad libitum. Drinking water was acidified with 0.7 ml concentrated HCl/l. Temperature was 22°C, humidity in the experimental environment was not regulated. Mice were permitted two weeks to acclimatize before entry into an experiment.

Cell lines

Two established human prostatic carcinoma cell lines, PC3 and DU145, were used in these studies. The tumor lines are derived from metastatic lesions in bone (PC3) (22) and brain (DU145) (23) from two different patients who suffered from prostatic cancer. Both lines were obtained from the American Type Culture Collection (ATCC No. 1435-CRL and 81-HTB). Cells were maintained in complete medium consisting of RPMI 1640 culture medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 250 nM dexamethasone, and 20 mM HEPES (GIBCO Ltd., Paisley, Scotland). Stock cultures of the cells were maintained in monolayer culture at 37°C in 6% CO_2 in air with 100% humidity.

Drugs

Human IFN-alpha, IFN-gamma and TNF, kindly supplied by Boehringer Ingelheim, Alkmaar, The Netherlands, were produced in Escherichia coli. The specific activities of IFN-alpha and IFN-gamma were 3.2×10^8 units/mg protein and 2×10^7 units/mg protein, respectively. Specific activity was measured by inhibition of encephalomyocarditis (EMC) virus replication in A549 cells (human lung myeloma cell line) with reference to the National Institute of Health (NIH) IFN-alpha standard Go 23-901-527 and the NIH IFN-gamma standard Gg 23-901-530. The purity of both IFN's was > 98% as determined by SDS polyacrylamide gel

electrophoresis and the amount of endotoxin was less than 1.0 ng/mg protein for IFN-alpha and less or equal to 0.5 ng/mg protein for IFN-gamma as based on the limulus amoebocyte lysate assay. The specific activity of TNF determined in the presence of Actinomycin D was 6 x 10^7 Units/mg protein (L-929 cytotoxicity assay). The purity was > 99% as determined by SDS polyacrylamide gel electrophoresis and it contained 1.0 ng or less endotoxin/mg protein based on the limulus amoebocyte lysate assay. The drugs were dissolved in the accessory dissolvent and diluted with unsupplemented RPMI medium. After dilution, drugs were divided in aliquots of 1.2 ml and stored at -80°C until use.

In vivo drug testing and monitoring

In order to propagate tumors in vivo, 10^6 cells of in vitro passage 20 for the PC3 and 4 x 10^6 cells of passage 64 for DU145 were injected subcutaneously (s.c.) in five mice per line; both lines showed a tumor take of 100%. Tumors were maintained by serial transplantation. After excision from a tumor-bearing animals, and removal of normal and necrotic tissue, tumor tissue was cut into pieces of 1-2 mm cubes and implanted s.c. in the right flank of recipient animals. For the in vivo treatment protocols we used the fourth to seventh transplant generation for the PC3 and the second to fifth transplant generation of the DU145. IFNs and TNF were administered s.c., peritumorally, three and five times per week respectively, in a total injection volume of 200 μl. Combinations of IFNs or IFN and TNF were given as a single injection. Control mice received unsupplemented RPMI medium three times per week. Treatment started 24 hours after tumor implantation. Five mice were included in each group; experiments were carried out at least twice.

Tumor growth was followed by measurements of tumor size according to the method of Janik et al. (24) by the same technician throughout each experiment. Two times per week length (l), width (w), and height (h) were measured by using a slide caliper. No correction was made for skin thickness. The tumor volume (V) was calculated from the formula

$$V = 0.5236 \times l \times w \times h.$$

Statistical analysis

For each mouse, the tumor volume was estimated 57 days after tumor implantation by linear regression on the logarithms of the tumor volumes throughout this period. For each tumor line, the influence on the estimated tumor volumes by the different treatment regimens was measured by analysis of variance. Duplo experiments gave similar results ($p = 0.89$ for the PC3 and $p = 0.62$ for the DU145). The p-values in this paper were obtained by two-side t-tests (F-tests). Results were considered significant if they gave rise to a family-wise error of less than 0.05 according to Hochberg's modification of the Bonferroni inequality (25).

The effect of treatment A is defined as $E_A = V_{control}/V_A$ and the effect of

treatment B as $E_B = V_{control}/V_B$, where V_A, V_B, and $V_{control}$ are the estimates for the tumor volumes of treatment A, treatment B or control group respectively. Additivity is defined (26) as $E_{A+B} = E_A \times E_B$ (i.e. $\log E_{A+B} = \log E_A + \log E_B$). A combination of two treatments is synergistic when the effect of the combination (E_{A+B}) is significantly greater than the product of the effects of the separate treatments.

RESULTS

In order to test the effects of cytokines against hormone refractory prostatic cancer a dose finding study was performed with PC3-bearing mice. From these studies we concluded that a dose range of 150 - 1,500 IU for each IFN is the most appropriate for combination drug testing. These doses are equivalent to 0.5 - 5 ng IFN-alpha and 8 - 80 ng IFN-gamma per gram body weight. Treatment with the highest dose of IFN-alpha (25 ng/g) resulted in a complete growth inhibition of all treated mice, whereas the highest dose of IFN-gamma (400 ng/g) could not inhibit tumor growth completely (Table I).

For initial studies with TNF we choose the dose used by Sherwood et al. (16) and a ten times higher dose; 50 and 500 ng/g five times per week. No cachexia was observed during TNF treatment. However, tumors developed in all mice in these preliminary studies. Both doses were used for further studies.

Effect of cytokine monotherapy on prostatic xenograft growth

The antitumor effects of IFN-alpha against the PC3 tumor are depicted in figure 1A. IFN-alpha 0.5 and 5 ng/g body weight have significant antitumor effects; 5 ng/g gives rise to a complete growth inhibition in 11 out of 15 mice (Table I). IFN-alpha has, in contrast to the very effective growth inhibition in the PC3 tumor, no significant effects against the DU145 tumor (fig. 2A).

IFN-gamma monotherapy is less effective than IFN-alpha and could not significantly suppress DU145 tumor growth (fig. 2B, Table I). Likewise, PC3 tumor growth was not inhibited by 8 ng/g bodyweight IFN-gamma. Application of 80 ng/g, however, had significant antitumor activity (fig. 1B).

On basis of units per gram body weight administered it is possible to compare the effectivity of the two different IFNs. Comparison of IFN monotherapies against the PC3 tumor showed that IFN-alpha 0.5 ng/g had a significantly stronger antitumor effect than IFN-gamma 8 ng/g ($p < 0.001$). The higher IFN-alpha dose was also more effective than 80 ng/g IFN-gamma ($p < 0.001$).

Table I

Cytokine mono- and combination therapy against PC3 and DU145 tumors. Statistical analyses and number of tumors growing per treatment group.

	PC-3		DU145	
Treatment[a]	p-value[b]	tumor growth[c]	p-value	tumor growth
control	-	17/17	-	10/10
alpha-IFN 0.5	<0.0001*	10/14	0.088	9/10
alpha-IFN 5	<0.0001*	4/15	0.077	10/10
alpha-IFN 25	ND	0/5		ND[d]
gamma-IFN 8	0.11	13/15	0.046	8/9
gamma-IFN 80	<0.0001*	12/14	0.017	8/10
gamma-IFN 400	ND	5/5		ND
TNF 50	0.0001*	11/15	0.04	9/10
TNF 500	<0.0001*	12/15	<0.0001*	6/10
alpha 0.5/gamma 8	<0.0001*	2/10	0.019	9/10
alpha 0.5/gamma 80	<0.0001*	0/10	0.041	9/10
alpha 5/gamma 8	<0.0001*	0/10	0.022	9/10
alpha 5/gamma 80	<0.0001*	2/10	0.13	10/10
alpha 0.5/TNF 50	<0.0001*	3/10	0.002	8/10
alpha 0.5/TNF 500	<0.0001*	3/10	0.0003*	8/10
alpha 5/TNF 50	<0.0001*	0/10	<0.0001*	7/10
alpha 5/TNF 500	<0.0001*	0/10	<0.0001*	6/10
gamma 8/TNF 50	0.016	10/10	0.044	9/10
gamma 8/TNF 500	<0.0001*	7/10	<0.0001*	8/10
gamma 80/TNF 50	<0.0001*	8/10	<0.0001*	7/10
gamma 80/TNF 500	<0.0001*	5/10	0.0003*	6/9

[a] treatment in ng/g body weight
[b] t-test comparing treatment vs. control
[c] number of mice with tumor/total number of mice
[d] not determined
* significant according to Hochberg's modification of the Bonferroni inequality

TNF monotherapy with 50 ng/g, five times per week, was effective againstthe PC3 tumor (fig. 1C), but not against the DU145 tumor (fig. 2C). The highest TNF dose, however, had a clear growth inhibiting effect against both xenografts. Furthermore, it should be noted that TNF 500 ng/g is the only monotherapy tested against the DU145 tumor, which resulted in a growth inhibition significantly different from the untreated growth control. None of the mice died due to the TNF treatment.

Figure 1

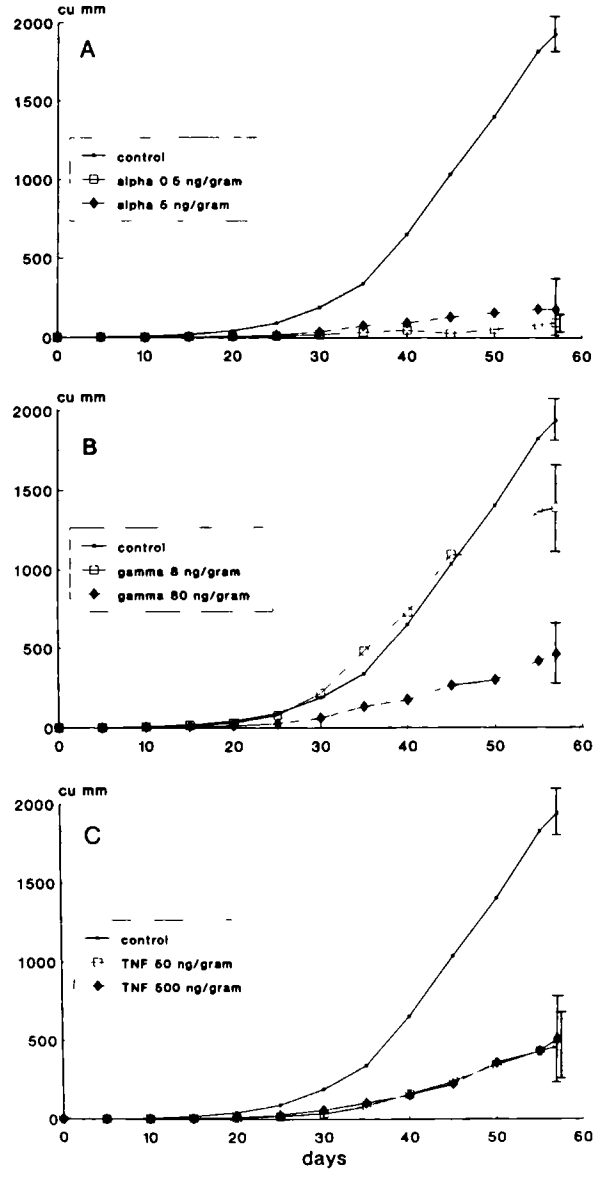

Figure 1. Antitumor effects of cytokine monotherapy against the PC3 tumor. Drugs were administered s.c., peritumorally; treatment started 24 hours after tumor implantation. Ten mice were included in each treatment group. Bars indicate standard error of the mean (SEM). A: IFN-alpha monotherapy, 0.5 or 5 ng/g body weight, 3 times per week. B: IFN-gamma monotherapy, 8 or 80 ng/g body weight, 3 times per week. C: TNF monotherapy, 50 or 500 ng/g body weight, 5 times per week.

Figure 2

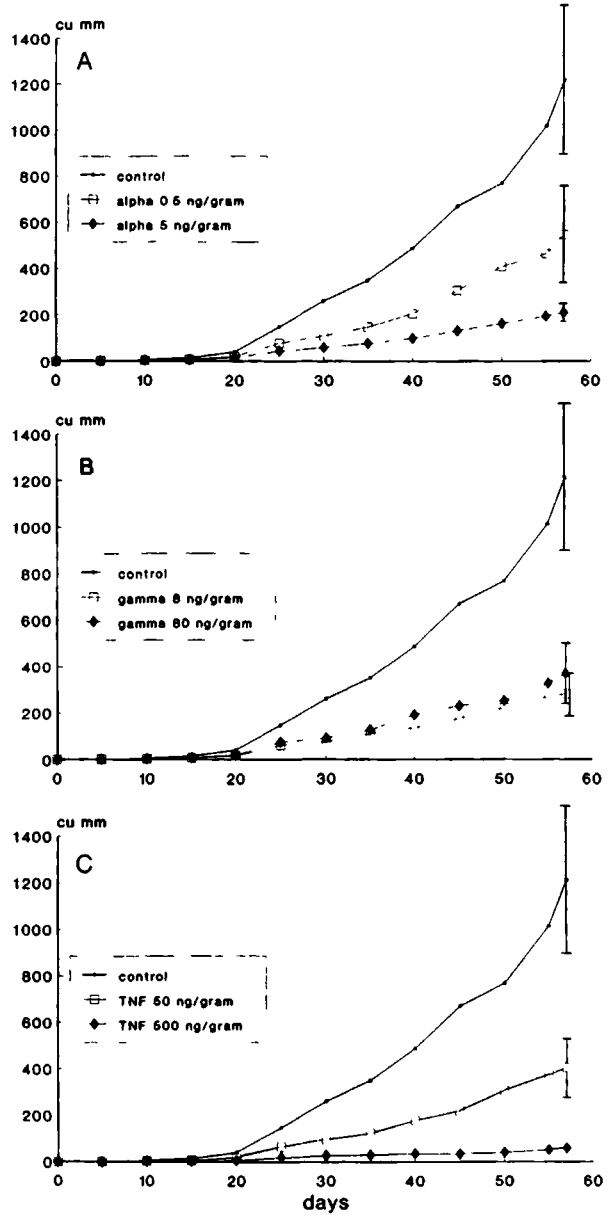

Figure 2. Antitumor effects of cytokine monotherapy against the DU145 tumor. Drugs were administered s.c., peritumorally; treatment started 24 hours after tumor implantation. Ten mice were included in each treatment group. Bars indicate SEM. A: IFN-alpha monotherapy, 0.5 or 5 ng/g body weight, 3 times per week. B: IFN-gamma monotherapy, 8 or 80 ng/g body weight, 3 times per week. C: TNF monotherapy, 50 or 500 ng/g body weight, 5 times per week.

IFN-alpha and -gamma combination therapy

The pronounced antitumor effect of IFN-alpha alone against the PC3 tumor makes it difficult to prove synergism in combination with another cytokine. All combination regimens of both low- and high-dose IFN-alpha and IFN-gamma were very effective against PC3 tumor growth ($p < 0.0001$); only four out of 40 mice developed a tumor during the treatment period (fig. 3A, Table I). In one of the duplo experiments mice were not sacrificed at the end of the treatment period, but were kept alive. After 8 weeks in 13 out of 20 mice a tumor started to grow, whereas in the IFN-alpha monotherapy regimens all mice developed a tumor (data not shown).

Unlike the PC3 tumor, combinations of IFN-alpha and IFN-gamma have very limited effects in the treatment of the DU145 tumor. Effects of none of these combinations could reach the level of significance (fig. 3B).

Figure 3

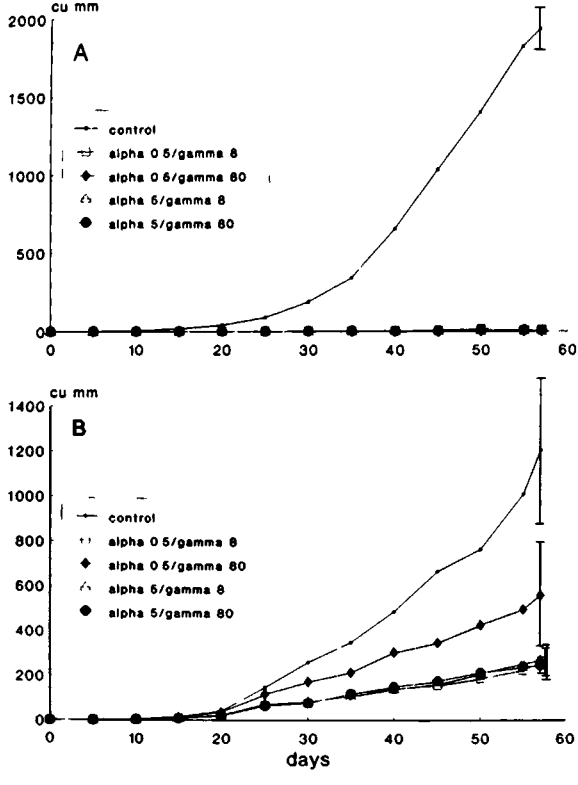

Figure 3. Antitumor effects of IFN-alpha and -gamma combination therapy. Drugs were administered s.c., peritumorally, 3 times per week; treatment started 24 hours after tumor implantation. Ten mice were included in each treatment group. Bars indicate SEM. A: IFN-alpha 0.5 or 5 ng/g body weight and IFN-gamma 8 or 80 ng/g combination therapy against the PC3 tumor. B: IFN-alpha 0.5 or 5 ng/g and IFN-gamma 8 or 80 ng/g combination therapy against the DU145 tumor.

IFN-alpha and TNF combination therapy

Treatment of PC3-bearing mice with combinations of IFN-alpha 5 ng/g plus both doses of TNF resulted in complete growth inhibition (fig. 4A). After cessation of treatment, these mice were kept alive and no tumors developed after a period of at least 8 weeks. These combinations of IFN-alpha with TNF are, therefore, more effective than the combinations with IFN-gamma in which tumors did develop after the treatment period. In the combinations with IFN-alpha 0.5 ng/g plus TNF 50 or 500 ng/g, eight out of ten mice developed a tumor 8 weeks after cessation of treatment. IFN-alpha 0.5 ng/g plus TNF 500 ng/g and both combinations with IFN-alpha 5 ng/g showed significant growth inhibitions of the DU145 tumor compared to the control group (fig. 4B). Complete growth inhibition, however, could not be achieved with any of these combinations. The best effect was found with the combination of IFN-alpha 5 ng/g and TNF 500 ng/g. Due to the great effect of TNF 500 ng/g monotherapy this combination was not synergistic.

Figure 4

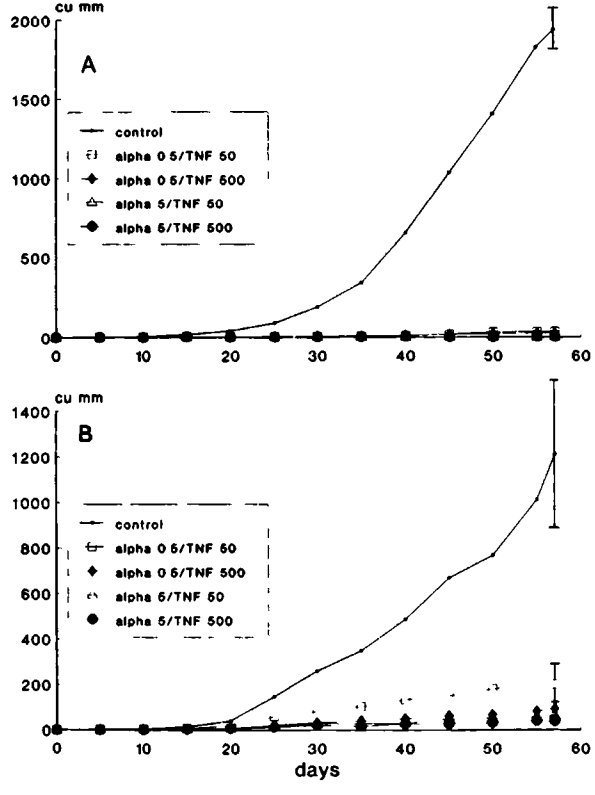

Figure 4. Antitumor effects of IFN-alpha and TNF combination therapy. Drugs were administered s.c., peritumorally, IFN-alpha 3 times and TNF 5 times per week; treatment started 24 hours after tumor implantation. Ten mice were included in each treatment group. A: IFN-alpha 0.5 or 5 ng/g body weight and TNF 50 or 500 ng/g combination therapy against the PC3 tumor. B: IFN-alpha 0.5 or 5 ng/g and TNF 50 or 500 ng/g combination therapy against the DU145 tumor.

Gamma-IFN and TNF combination therapy

In the treatment of the PC3 tumor, all combinations of gamma-IFN and TNF, except for the combination of both lowest doses, had significantly growth inhibiting effects compared to the growth control (fig. 5A). Synergistic effects, however, were not observed. The combination of gamma-IFN 8 ng/g and TNF 500 ng/g, for example, is significantly more effective than IFN-gamma monotherapy, but not more effective than TNF 500 ng/g alone. Against the DU145 tumor three out of four gamma-IFN and TNF combinations produced significant growth-inhibiting effects, synergistic effects, however, were not observed (fig. 5B).

Figure 5

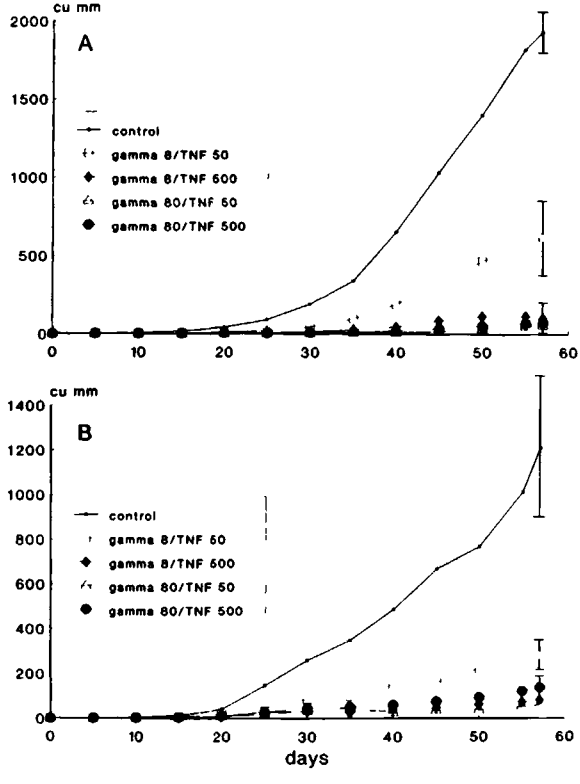

Figure 5. Antitumor effects of IFN-gamma and TNF combination therapy. Drugs were administered s.c., peritumorally, IFN-gamma 3 times per week and TNF 5 times per week, treatment started 24 hours after tumor implantation. Ten mice were included in each treatment group. Bars indicate SEM. A: IFN-gamma 8 or 80 ng/g body weight and TNF 50 or 500 ng/g combination therapy against the PC3 tumor. B: IFN-gamma 8 or 80 ng/g and TNF 50 or 500 ng/g combination therapy against the DU145 tumor (combination IFN-gamma 80 ng/g and TNF 500 ng/g, n = 9).

DISCUSSION

Prostatic cancer is frequently in a locally advanced stage at presentation, making radical treatment such as surgery or radiotherapy ineffective (3). For almost 50 years, since the pioneering efforts of Huggins and Hodges, hormonal therapy has been the major treatment modality for metastatic prostatic cancer (4). After initial response, the patient will inevitably develop a tumor which is hormonally unresponsive and resistant to current therapeutic modalities. Therefore, it would be useful to develop new ways to treat hormone-resistant tumors.

In vitro studies with IFN-alpha and -beta showed antiproliferative efficacy of both drugs against the PC-3 and DU145 cell lines (12,13), IFN-beta being more effective than IFN-alpha (12). The androgen-dependent LNCaP cell line, however, was resistant to IFN-beta treatment (27). In vitro studies with TNF revealed controversial results. Sherwood et al. reported cytotoxicity toward all three prostatic cell lines (16), Fruehauf et al. also found sensitivity of the LNCaP cell toward TNF; PC3 cells, however, were resistant (17).

We have investigated the in vivo antitumor effects of IFN-alpha and -gamma and TNF against hormone-independent prostatic tumor lines. IFN-alpha monotherapy was very effective against the PC-3 tumor; no significant differences were found between the effects of 0.5 and 5 ng/g body weight. Alpha-IFN 25 ng/g, however, inhibited tumor growth completely. Gamma-IFN 80 ng/g had significant antitumor effects, whereas 8 ng/g could not reduce tumor growth. Also, the highest dose IFN-alpha appeared to be more effective against the DU145 tumor although this was not significant. Both gamma-IFN doses mediated similar, not significant, growth-inhibiting effects. The antitumor effects of the IFNs can best be described as dose-dependent in the dose range tested. This in contrast to the so called bell-shaped response curves we have found for alpha- and gamma-IFN against some of eight different renal tumor xenografts (28). Clinical trials with IFN-gamma in renal cell carcinoma also revealed an optimal treatment dose for these tumors. Aulitzky and co-workers showed that a relatively low dose of gamma-IFN, 100 μg (2×10^6 IU) once weekly is biologically more active than higher doses. The low dose resulted in a nearly maximum rise of neopterin and ß2-microglobulin levels in the serum with minimal side effects, whereby the increments in these markers were predictive for response (29).

TNF 500 ng/g was the only monotherapy which had significant antitumor effects in the DU145 tumor. Growth of the PC3 tumor was significantly inhibited by both TNF doses, which is in contrast to the in vitro data of Fruehauf et al. (17). Sherwood et al. also showed in vivo growth inhibition of the PC3 by intravenous administered TNF in a dose of 50-100 μg/kg every other day (16).

In our studies we administered the cytokines peritumorally. In clinical trials, however, treatment is usually given systemically, which will result in lower local drug

levels. Unlimited increase of cytokine administration is not possible due to toxicity, especially of TNF (30). In the studies presented here, we have shown that combinations of cytokines can produce significant antitumor effects with lower cytokine concentrations; e.g., low TNF doses in combination with IFN-alpha are more effective than high dose TNF monotherapy against the PC3 tumor. Another possibility to overcome the significant host toxicity of cytokines could be the use of liposome-encapsulated cytokines. TNF administered in this way retains its immunomodulatory activity, but has significantly lower toxic inflammatory effects when compared to free TNF in vivo (31). Liposome-encapsulated IFN-alpha retained its antitumor effects against bladder tumor cell lines in vitro (32). Furthermore, small changes in the TNF molecule can make it much less toxic (33).

Heterotransplantation of human tumors into nude mice provides a model in which to study the antitumor activity of new drugs on human tumors in vivo (34). Due to the congenital absence of the thymus, cell-mediated immune functions in nude mice are severely impaired. The animals lack one of the important effector cells, the (cytotoxic) T-cell, and therefore, only the direct effects of the cytokines can be studied. Moreover, both the antiproliferative and immunomodulatory effects of IFNs are species-specific. Human IFN-alpha and IFN-gamma have no effects on the mouse immune system as measured by NK cell or macrophage activity (35,36). This makes it more unlikely that indirect effects contribute to the antitumor effects.

A possibility in studying the complete spectrum of cytokine-mediated effects, i.e., direct and indirect host-mediated effects, could be the use of severely combined immunodeficiency (SCID) mice in which the human immune system can be partly reconstituted (37,38). Another possibility is the use of animal tumors in syngeneic animals. We have investigated the antitumor effects of rat gamma-IFN and human TNF in Dunning rat prostatic tumors and found limited activity of cytokine monotherapy but synergistic effects of the different combination treatments (39).

Conclusion

Most combination treatments resulted in significant growth inhibitions of the DU145 tumor. Combinations were also very effective against the PC3 tumor, e.g. combinations with IFN-alpha give rise to tumor growth in 10 out of 80 treated mice. We, therefore, conclude from our data that cytokine combination treatment can provide a new approach in the treatment of hormone-escaped prostatic tumors.

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CHAPTER VII

ACCUMULATION OF GENETIC CHANGES EVALUATED IN A NEW RAT TRANSITIONAL CELL CARCINOMA PROGRESSION MODEL

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ABSTRACT

A cohort of 300 ACI rats was kept under standard laboratory conditions. After 30 months or upon natural death complete autopsy was performed. In the genitourinary tract 4 kidney and 5 bladder tumors were found. Two of these bladder tumors, RBT323 and RBT157, are serially transplantable. In the 5th transplant generation the RBT323 tumor becomes metastatic to the lungs in more than 90% of the animals. The metastatic ability of the RBT157 tumor changed from low to intermediate (50% of the rats have lungmetastases) in the 4th passage. Histologically, the initial passages of the RBT323 and 157 tumors are grade II transitional cell carcinoma. The histological pattern of the RBT157 tumor remains essentially unchanged, whereas the RBT323 tumor changes into a grade III tumor in the third passage. Electronmicroscopical studies revealed oblong elliptical and round vesicles lined by an asymmetrical unit membrane in the tumor cells, which stresses the urothelial origin of the tumors. Immunohistochemically both tumors show expression of cytokeratin 5,7,8 and 18. The progression of the tumors to a metastatic phenotype, however, was not associated with a specific change in the morphological characteristics. Cytogenetic analysis of different passages showed that both tumors are peridiploid with few marker chromosomes. In subsequent passages an accumulation of marker chromosomes was found. Furthermore, in both tumors a loss of chromosomes was found which maintained during tumor progression. Flow cytometric analysis showed an aneuploid DNA content in the initial passages of the RBT323 tumor, the DNA content of subsequent transplant generations, however, was diploid, which is similar to the RBT157 tumor.

Two independently arisen rat tumor lines that represent the progression of bladder cancer are described. This model system may be helpful in the identification of specific markers associated with the progression of bladder cancer.

INTRODUCTION

Estimates of new cancer cases in the United States show that cancer of the bladder is the fourth most prevalent cancer in men and the ninth among female patients. Bladder cancer is about three times as common in men as in women. The estimates for 1990 are 36,000 new cases in males and 13,000 new cases in females in the USA. Bladder cancer death rates have been fairly constant the last 60 years (1,2). Recurrence rates vary from 30 per cent of a solitary papillary tumor to more than 90 per cent in some cases of multiple tumors. 5 to 30 per cent of all cases exhibit progression of disease (3). In general tumor progression is associated with large tumor size, high stage, high grade, multiplicity of tumors, vascular or lymphoid invasion and concurrent carcinoma in situ (3,4). More importantly, recent studies indicated involvement of specific chromosomal changes in tumor progression of TCC. In low-grade, non-invasive TCC trisomies for chromosome 1,7 and 11 and monosomy for chromosome 9 were found, with progression of the tumors the number of chromosomal aberrations increased (5). Allelic losses were found for chromosome 9, 11 and 17 (6). The greatest frequency was seen for chromosome 9q (67% of the informative cases) and 17p (63% of the informative cases). The authors concluded, after having investigated a greater number of cases, that the loss of chromosome 17 is a late event in tumor progression and can distinguish between low and high grade TCC (7).

An important restriction in the study of human bladder cancer is the lack of available tumor tissue for experimental purposes. Especially tumor material, truly representative for the progression from low to high stage TCC is very difficult to obtain. The tumor volumes obtained from routine biopsies and cystectomies are often small and damaged due to intra-operative or prior treatment. Many important factors, not specific for bladder cancer, cannot be controlled with clinical material, including genetic differences, age, time of initial onset of the disease and distribution, variability of cell types etc. To overcome these problems a variety of animal models for bladder cancer have been developed (8,9).

After heterotransplantation of human bladder cancer tissue into immune-deprived and nude mice some short term and serially transplantable xenograft lines have been established. No correlation has been found between the success of xenografting and the grade of the 'donor' tissue (9,10). Russell et al demonstrated substantial heterogeneity within and between different TCC xenografts. Studies with these lines may help to explain the diverse natural history of bladder cancer (12).

Animal tumor models can be divided in carcinogen-induced and spontaneous tumor models. A number of potent carcinogens can produce bladder neoplasms in laboratory animals (8,9,13). In most induced tumors concurrent squamous metaplasia occurs and most tumors consist of both poorly differentiated transitional cell carcinoma (TCC) and squamous cell carcinoma (SCC) (13,14). Few spontaneous

bladder tumor models are available up to now. The Brown Norway rat has a high incidence of spontaneously occurring urothelial tumors of the bladder and ureter, 35% of the male and 3% of the female rats developed bladder tumors, which are transplantable in syngeneic rats (15). The invasive tumors consisted of transitional epithelium, but also areas of squamous metaplasia were observed, SCC, however, was not common. In the Nb rat a single transplantable transitional cell tumor has been described (16). In DA/Han rats 54% of male and 14.4% of female rats develop spontaneous urinary bladder tumors (17). The incidence increases with age of the rats, also for induced carcinomas an increased risk of urinary bladder carcinogenesis with age has been reported (18). The most frequent tumors found in the DA/Han rats were solid type transitional cell carcinomas; 0.3% of the male and 9.6% of the female rats had SCC (17).

ACI rats are known for their spontaneous genito-urinary carcinomas, especially prostatic carcinomas, but TCC has been described also (18,19). In this paper we present the characterization of two new transplantable TCC in ACI rats. Furthermore, these lines are evaluated for phenotypic and genetic changes occurring in the progression of the lines during serial transplantation.

MATERIALS AND METHODS

Animals

For the initial tumor-finding study, 300 male ACI/SegHSD (Harlan Sprague, Inc., Indianapolis, IN) were housed in an environmentally controlled room and were provided with RMH-TM food (Hope Farms BV., Woerden, The Netherlands) and water ad libitum. Upon natural death complete autopsy was performed. The urinary bladder and prostate were excised in toto and fixed for at least 24 hours and embedded in paraffin. Sections were cut transversely through the midportion of the bladder, and four 5 μm sections were taken from each half to adequately sample the entire bladder and prostate; these sections were stained with hematoxylin and eosin.

Tumor Transplantation

Primary tumors and tumors from subsequent passages were excised from tumor-bearing animals, after removal of normal and necrotic tissue, the tumor tissue was cut into pieces of 20 mg. An 0.5 cm incision was made in the right flank of recipient animals, and after separating the subcutaneous tissue by blunt dissection, the tumor fragment was placed subcutaneously (s.c.). The incision was closed using skin clips (7.5 x 1.75 mm, Aesculap, Germany). In this way a tumor take of 100% was achieved. Per passage at least 6 rats were implanted. Tumor growth was followed by measurements of tumor size according to the method of Janik et al.

(21) by the same measurer throughout each experiment. At least once per week length (l), width (w) and height (h) were measured using a slide caliper. No correction was made for skin thickness. The tumor volume (V) was calculated from the formula V = 0.5236 x l x w x h. Per animal, the paired serial measurements of days post-tumor inoculation (x-axis) and log tumor volume (y-axis) were plotted to reveal the time period of exponential growth. By means of linear regression the best lines were fitted. The slopes of the regression lines represent the growth rate, the inverse of the slopes represent the tumor doubling times.

Light and Electron microscopy

When the subcutaneously growing tumors reached a size of 5-10 cm^3, rats were sacrificed with an ether overdose and complete necropsy was performed. Both lungs and representative pieces of the tumor were routinely fixed in 4% buffered formalin for at least 24 hours and embedded in paraffin. Others organs were grossly inspected for metastases and if suspicious also fixed in formalin. To adequately sample the tumors and the lungs four 5 μm sections were taken and stained with hematoxylin and eosin. Tumors were assessed according to Squire (22) and Koss et al. (23).

For electron microscopy representative parts of the tumors were cut into small pieces and prefixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 C, and postfixed in 0.1 M paladebuffer. After washing in the same buffer and degradation in graded ethanols, the specimens were embedded in Epon 812. Thin sections were double-contrasted with uranyl acetate/lead citrate and examined in a Philips EM 300 (24).

Immunohistochemistry

For immunohistochemical studies we used the indirect immunofluorescence technique as described before (25). Frozen sections of the tissues (6 μm) were cut on a cryostat and air-dried before fixation in methanol (5 min., -20 ^{0}C) and acetone (3 times 5 sec.). After being washed in phosphate-buffered saline (PBS) for 10 min, the sections were incubated with 20% normal rat serum (NRS) in PBS at room temperature (30 min), and subsequently with the primary antiserum for 60 min. After repeated washing in PBS (3 times 10 min) the rabbit anti-mouse conjugated to peroxidase (DAKOpatts, Denmark) diluted 1:100 (v/v) in PBS with 20% NRS was applied for 30-45 min. After repeated washings in PBS (3 times 10 min), peroxidase activity was detected with 3-3'diaminobenzidine (DAB; 6 mg/10 ml, 0.65% imidazole in PBS) and hydrogen peroxide to a final concentration of 0.01%. After incubation for 5 min. and extensive washing with tap water, the slides were counterstained with hematoxylin, and mounted with permount (Fisher Scientific, New, Jersey, USA).

The following antibodies directed against intermediate filament proteins were used in this study:

1. The mouse monoclonal antibody RCK 102, directed against keratins 5 and 8 (nomenclature according to Moll (26)). This antibody stains virtually all epithelial tissues, but not nonepithelial tissues (27).
2. The mouse monoclonal antibody RCK 105, directed against keratin 7 and reactive with a subgroup of glandular epithelial tissues (27).
3. The mouse monoclonal antibody RGE 53 directed against keratin 18, which specifically recognizes columnar epithelial cells from digestive, respiratory, and urogenital tracts, endocrine and exocrine tissues, and mesothelial cells. No significant reaction is normally found in squamous epithelial or nonepithelial tissues (28).
4. The mouse monoclonal antibody RKSE 60, directed against human skin keratin 10 and specific for keratinizing stratified squamous cells. No reaction is found with this antibody in columnar epithelial cells, nonkeratinizing squamous cells, or nonepithelial cells (28).
5. The mouse monoclonal antibody RV 203 is specific for vimentin (29).
6. The mouse monoclonal antibody RD 301 is specific for desmin (30).

Preparation of single cell suspensions

When tumors reached a size of 5-10 cm^3 the rats were sacrificed and tumor material was suspended in RPMI 1640 tissue culture medium (Gibco, Paisley, UK). After careful removal of normal tissue and areas of tumor necrosis, tumors were cut into pieces and minced with scissors into a 300 μm metal sieve and continuously washed with RPMI 1640 solution into a petri dish. The minced tumor tissue was passed through a 40-70 μm nylon filter (Ortho Diagnostics, Beerse, Belgium) to obtain a single cell suspension. This cell suspension was used for flowcytometric analysis and/or growth in the colony forming assay.

Flow Cytometry

The single cell suspension was centrifuged at room temperature at 400g for 5 min, after which the supernatant was discarded but fluid sticking to the wall of the tubes was allowed to cover the pellet, as described before (31). The pellet was vortexed vigorously and 70% ethanol (-20^{0}C) was added rapidly under constant vortexing. The final concentration was about 3 x 10^6 cells/ml. At this stage the cells could be stored at -20^{0}C. For DNA measurements 1 x 10^6 cells were resuspended in 1 ml of a propidium iodide (PI) solution (20 mg/l A grade in 150 mM sodium phosphate buffer, pH 7.4, Calbiochem-Boehringer, La Jolla, CA). To 1 ml of this cell suspension, 0.1 ml of a stock solution of RNAse (1% type A in sodium phosphate buffer, Sigma) was added and subsequently incubated for exactly 10 min at 37^{0}C. Then the cell suspension was filtered through a 49 μm filter (Ortho). Cells were kept in the dark prior to FCM analysis.

DNA measurements were performed using a Cytofluorograph 50H (Ortho Instruments, Westwood, MA). PI was excited at 488 nm with a argon ion laser (Spectra physics, Mountain View, CA). Fluorescence was measured using a 630 nm longpass filter. All data were recorded as 2048 channel histograms and stored in a PDP 11/34 computer (Digital Equipment Corporation, Marlboro, MA) for subsequent data analysis. For ploidy measurements chicken red blood cells (CRBC) were used as internal standard. A mixture of CRBC and rat lymphocytes served as an external standard. The degree of DNA content aberration is expressed by the DNA index (DI), which is the ratio of the mean of the relative DNA content of the G0/G1 cells of the tumor divided by the mean of the relative DNA measurements of the diploid G0/G1 rat lymphocytes (32). Diploid cells have, by definition, a DNA index of 1.0. Tumors were qualified FCM (near)-diploid (DI = 0.9-1.1), FCM aneuploid (DI = 1.2-1.7; > 2.2) or FCM tetraploid (DI = 1.8-2.2). The coefficient of variation of the Go/G1 peaks analyzed was between 2 and 6% (mean 4%).

Soft-agar culture

The single cell suspension was centrifuged at room temperature at 400g for 5 min after which the supernatant was discarded. Upon resuspension of the cell pellet in double enriched CMRL 1066 (Gibco, Paisley, UK), cell density and viability were determined by adding 15 μl trypan blue solution (25 mg in 5 ml 3% acetic acid) to 15 μl cell suspension and simultaneously counting colored and not colored cells using a Bürker Türk haemocytometer. For the detection of the in vitro growth potential a modified double layer soft agar culture method as originally described by Salmon and Hamburger (33) was used (34). Tumor cell suspensions were plated in the upper layer of the two-layer culture system in a range of 10,000 - 500,000 cells per dish. Cells were cultured immediately after preparation of the single cell suspension, growth potential was quantified using an Omnicon Fas II automated colony counter (Milton Roy Inc., Rochester, New York (35)).

Chromosomal Analysis

Single cell suspensions were made by mincing tumors with scissors in RPMI 1640 culture medium containing 10% fetal bovine serum (FBS) and colcemide (0.02 μg/ml). After incubation at 37 ^{0}C for 10 minutes the medium was replaced with 0.075 M KCl hypotonic solution containing colcemide (0.02 μg/ml) which was prewarmed to 37 ^{0}C. The suspension was incubated at 37 ^{0}C for 25 minutes and fixed with methanol:acetic acid (3:1). Chromosomal slides were prepared by dropping the cell suspension onto clean slides in a humid box. Thirty to 50 metaphases were counted for each tumor. Chromosomes were banded using the trypsin-Giemsa technique (36) and arranged according to the scheme of Satoh et al. (37). At least five G-banded metaphases were karyotyped for each tumor.

Etiology and growth characteristics

300 male ACI rats were kept under standard laboratory conditions, without influence of any known carcinogen. Approximately 150 animals died within 24 months, the remaining animals were sacrificed at 30 months. In the genitourinary tract 4 kidney tumors and 5 bladder tumors were found. In contrasts to other studies, no prostate tumors developed in these rats (18). All 5 bladder tumors were transplanted s.c. in syngeneic rats, 2 tumors appeared to grow under these conditions. These tumors were designated Rat Bladder Tumor 323 and 157 (RBT323 and RBT157). Both tumors were slowly growing (Table 1). Initially both tumors typically grew as a cyst, with papillary tumors inside this cyst. Due to this growth pattern the tumor doubling times of the first passages should be considered inaccurate. Upon further passaging the tumor acquired a more solid growth pattern. The tumor doubling time of the RBT323 tumor decreased from 13 days in second passage to 3.5-4 days in passage eight and subsequent passages (at time of submission RBT323 tumor is in transplant generation #20). The tumor doubling time of the RBT157 tumor, however, was almost not changed. In the first passage the doubling time is 11 days, subsequent passages have doubling times of 8-9 days (tumor presently in pass. #7).

The metastatic capacity of both tumors is low in the initial passages (i.e. less than 5% of the animals have microscopic lung metastases) (Table I). However, in passage 5 for the RBT323 tumor and passage 4 for the RBT157 tumor, both tumors acquire a higher metastatic potential especially to the lungs. More than 90% of the RBT323-bearing animals have microscopic and/or macroscopic lung metastases, occasionally an axillary or retroperitoneal lymphnode metastasis was found. No liver metastases were found. About 50% of RBT157-bearing animals show metastases to the lungs.

Cells of transplant generation 1, 12 and 13 of the RBT323 tumor and generation 1 and 4 for the RBT 157 tumor were tested for their in vitro growth potential. The trypan blue exclusion test usually showed a viability of 20%. 14 days after plating 500,000 RBT323 cells 250-300 colonies were formed in the dishes, i.e. a plating-efficiency of 0.06%. Cells of RBT157 passage 1 form 20 colonies, while cells of transplant generation 4 form 70 colonies after 14 days of culture (plating efficiency from 0.004 - 0.014%).

Table I

Tumor passage#	DNA-index[a]	Tumor doubling time[b]		metastases[c]
RBT323	1.759	ND		0/1
RBT323.p1	1.645	ND		0/6
RBT323.p2	0.916	13.43	(1.40)	0/8
RBT323.p3	0.999	11.81	(1.23)	1/10
RBT323.p4	0.976	5.95	(1.02)	0/9
RBT323.p5	1.110	5.69	(0.25)	3/7
RBT323.p6	1.017	4.35	(0.10)	11/13
RBT323.p8	0.999	3.84	(0.15)	15/15
RBT323.p10	0.970	3.68	(0.22)	9/9
RBT323.p12	1.101	4.16	(0.27)	19/22
RBT323.p13	1.024	3.26	(0.25)	6/6
RBT323.p15	1.002	4.29	(0.34)	5/6
RBT323.p6 (meta)	0.946	ND		
RBT157	1.151	ND		0/1
RBT157.p1	1.016	11.15	(0.82)	0/4
RBT157.p2	1.061	ND		1/7
RBT157.p3	1.017	10.50	(1.12)	2/9
RBT157.p4	1.067	8.19	(0.32)	5/8
RBT157.p6	1.049	9.46	(1.80)	2/6

[a] FCM (near)-diploid: DI = 0.9-1.1, FCM aneuploid: DI = 1.2-1.7; > 2.2) or FCM tetraploid: DI = 1.8-2.2
[b] Tumor doubling time in days, standard error of the mean in brackets
[c] Number of rats with lungmetastases/total number of rats implanted with respective tumor passage

Table I. Growth characteristics of rat bladder tumor lines

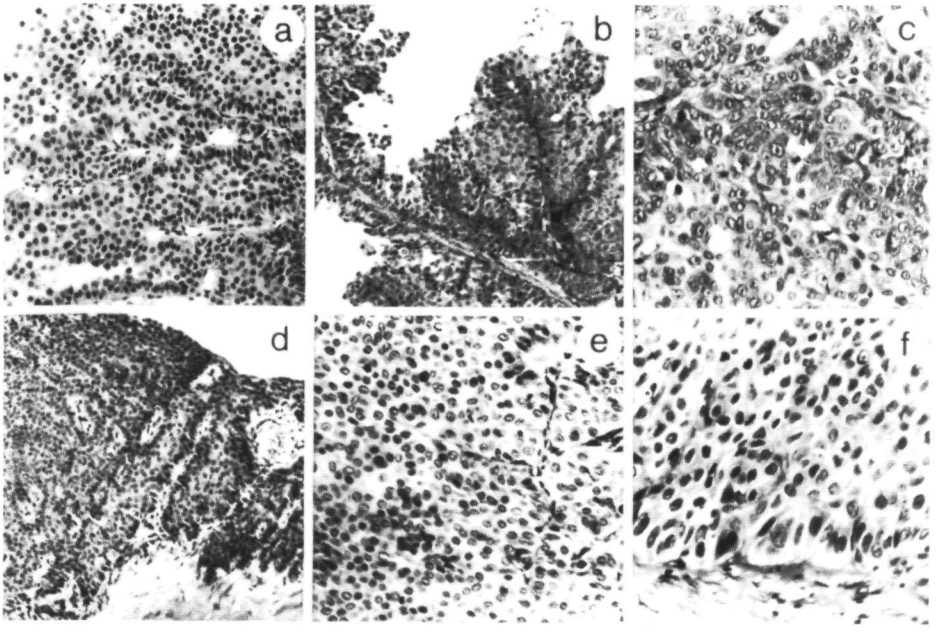

Figure 1. RBT323 (a-c) and RBT157 (d-f) tumors (H & E stain).
a: RBT323, original tumor (x 125), b: RBT323, first passage (x 125)
c: RBT323, passage #14 (x 250), d: RBT157, original tumor (x 125)
e: RBT157, first passage (x 250), f: RBT157, passage #4 (x 250)

Histology

The original RBT323 tumor shows a multicystic growth pattern with solid, trabecular and papillary regions (fig. 1). The cells are slightly disorderly arranged and the nuclei are moderately atypic. There is an increased mitotic activity, 0-2 mitoses per high power field (40x, HPF); grade II transitional cell carcinoma. The primary tumor exhibits no invasive growth. The wall of the cyst is lined by a 3-7 cell layers thick transitional epithelium with a superficial layer of umbrella cells. These cells are generally similar to the tumor cells.

The tumor of the first transplant generation has a similar growth pattern as the original RBT323 tumor, however, invasive growth into the subcutaneous tissues was found. In the subsequent transplant generations the anisokaryosis of the nuclei increases, nuclei become bigger and are more disorderly arranged, i.e. a grade III transitional tumor. This grade III growth pattern remains the same for all further transplant generations (upto generation 20 at the time). The RBT157 tumor has generally the same growth pattern as the RBT323 tumor, but has larger cells due to a larger cytoplasm (fig. 1). The nuclei are moderately atypical. In subsequent transplant generations the tumor remains a grade II transitional cell carcinoma. The mitotic index, however, increases to 0-3 per HPF. No squamous differentiation was found in both tumors.

Electron Microscopy

Generally the tumor cells of all passages demonstrate well developed nuclei and nucleoli, abundant organelles as well as distinct junctional complexes (fig. 2). The basal compartment cells of the first passage of the RBT323 tumor are surrounded by an intact basal lamina. In the intermediate layers many cells show additionally particles and clusters of glycogen, while other cells contain characteristic electron-dense membrane bound granules. In some foci well developed intermediated-sized filaments are present in the tumor cells. Numerous cells contain flattened discoid-like or fusiform membrane-bound vesicles, predominantly located in the juxtanuclear and the apico-luminal part of the cytoplasm. Close contact with the cell boundary is present. An adenomatous growth pattern of tumor cells can be observed and inter- as well as intracellular luminal formations are present. In the superficial cell layers several cells also demonstrate many flattened vesicles in the apical side of the cell. At higher magnifications the membranes of these vesicles are asymmetric with a thicker fuzzy luminal layer. Upon passaging of the RBT323 tumor the amount of junctional complexes apparently decreases with local absence of the basal laminae. The intracellular flattened vesicles change to more rounder shapes, some of them are obviously attached to the luminal cell membrane. Generally the first passages of the RBT157 tumor show the same growth pattern as the early passage of the RBT323 tumor. In contrast to the latter, the growth pattern shows no distinct changes in the later passages.

Immunohistochemistry

The normal rat bladder, the original tumors, and tumor tissues of the transplant generations 1 to 11 for the RBT323 tumor and 1 to 5 for the RBT157 were examined for their intermediate filament expression pattern by using monoclonal antibodies against keratins, vimentin and desmin.

The broadly cross-reacting keratin monoclonal antibody RCK 102 stains all urothelial cell layers of the normal bladder and the RBT157 tumors. In the RBT323 tumor, however, only the superficial "luminal" cell layer and the directly underlying cell layer are stained. Antibody RCK 105 also stains all cell layers of the normal rat bladder and the RBT157 tumors (fig. 3). In the primary RBT323 tumor this antibody stains all cell layers except most basal cells (only 10% of the basal cells stain) (fig. 3). In tumors of further transplant generations only the superficial cells and the directly underlying cell layer stain. Antibody RGE 53 reacts with the superficial and intermediate cell layers of the normal bladder and with 20% of the basal cells. In the primary RBT157 tumor all superficial and intermediate cell layers and about 30% of the basal cells are stained by RGE 53, in further transplant generations only the superficial and adjacent cells stain (fig. 3). In the primary RBT323 tumor all superficial and most intermediate cell cells react with RGE 53 (fig. 3). Upon transplantation of the tumor generally only superficial cells stain with

Figure 2

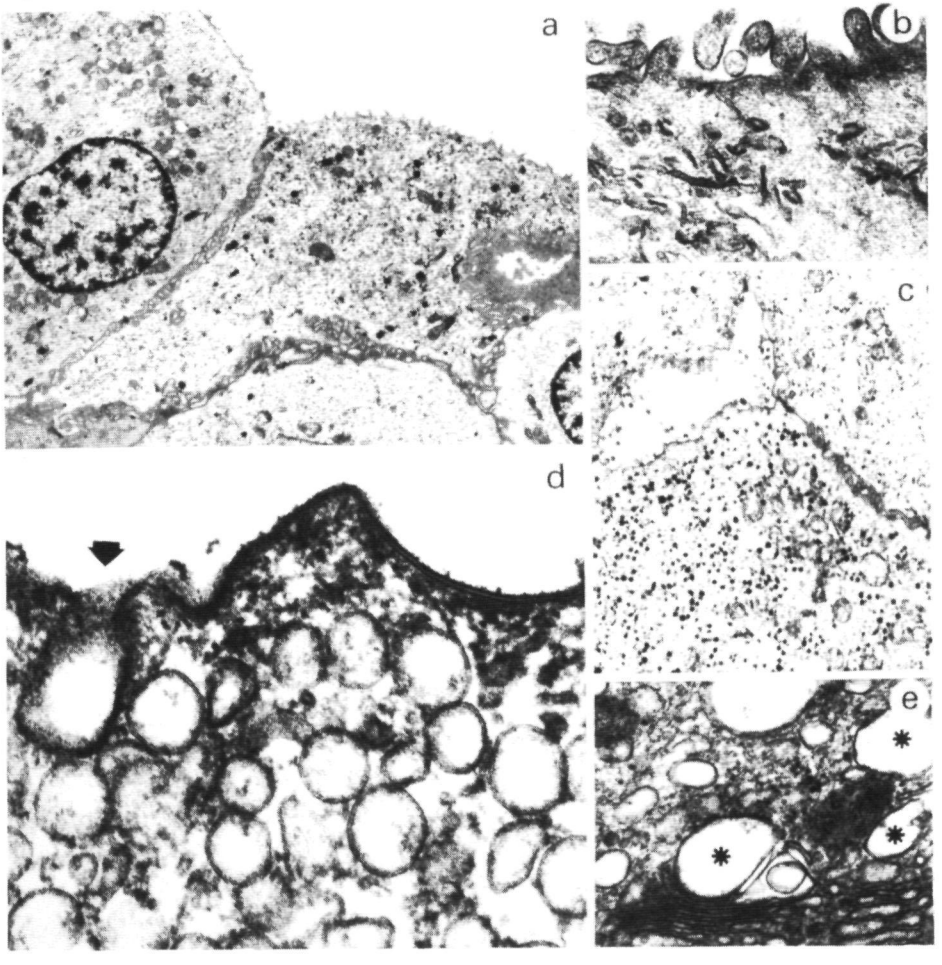

Figure 2. Electron micrographs of RBT323 tumor.
a: First passage (5,000 x), superficial tumor cells with abundant organelles are junctioned and exhibit lateral interdigitations, b: First passage (40,000 x), detail of a superficial cell with microvilli and profiles of flattened elliptical vesicles, c: First passage (7,000 x), strongly interdigitated, junctioned tumor cells, one tumor cell contains numerous endocrine granules, d: Passage #9 (120,000 x), high magnification of the apical part of a superficial cell, distinctly demonstrating the unit membrane of the vesicles lined by an inner fuzzy coating; arrow indicates attachment of a vesicle to the luminal membrane, e: Passage #9 (55,000 x), detail of a Golgi area showing early vesicle formation (asterisks); different stages of vesicular profiles are present.

RGE 53. From passage 1 to 6 the number of positive superficial cells decreases from 60% to 10%. This number increases, however, to 50% in the 11th passage, while in passage 9 all superficial cells are positive.

RKSE 60, an antibody recognizing markers for keratinization, is negative in the normal rat bladder and in the RBT157 tumors. In the RBT323 tumor occasionally cells in the superficial cell layer stain, the number of positive cells is usually only 1% or less. RV203 detects normal and tumor stroma, but never stained normal rat urothelium or tumor tissue of both tumors. Both normal rat bladder and tumor tissues did not stain with RD 301.

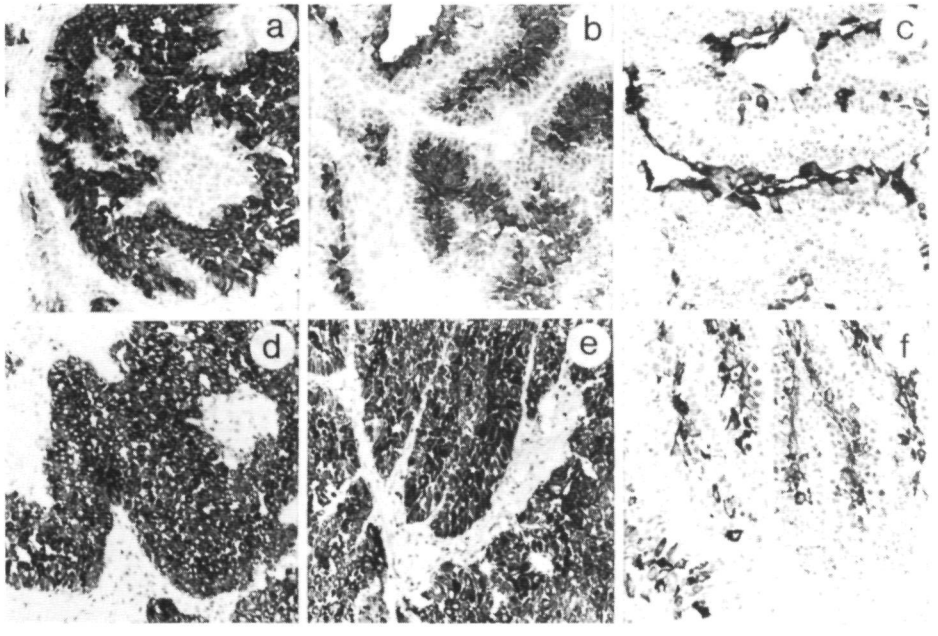

Figure 3. Immunoperoxidase staining pattern of frozen sections from RBT323 (a-c) and RBT157 (d-f) tumors. a: RBT323, original tumor, stained with anti-cytokeratin MoAb RCK102 (x 125), b: RBT323, passage #11, RCK102 (x 125), c: RBT323, first passage, RGE53 (x 125), d: RBT157, original tumor, RCK102 (x 125), e: RBT157, passage #4, RCK102 (x 125), f: RBT157, passage #4, RGE53 (x 125)

DNA measurements by Flow Cytometry

The DNA flow cytometric profiles of the original tumors and their subsequent serial passages are summarized in Table I. The original tumor and the initial passage of the RBT323 tumor (p1) show DNA aneuploidy, later passages demonstrate pure diploid DNA content (fig. 4). Lungmetastases of passage 6 have a diploid DNA content also. The DNA content of the original RBT157 tumor is near the 10% interval of diploidy and is, therefore, peridiploid. Subsequent passages of this tumor are diploid.

Figure 4

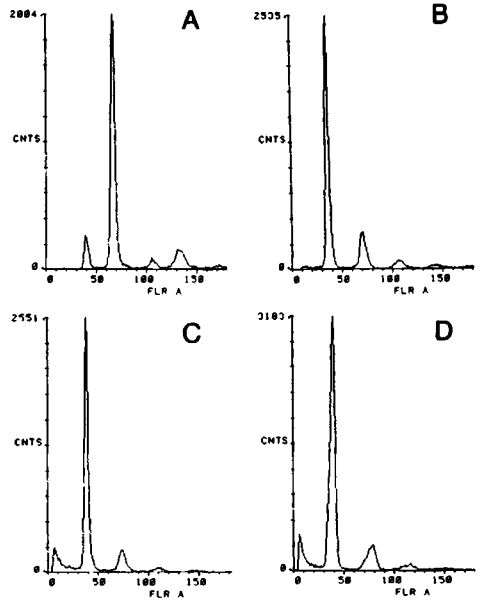

Figure 4. Flow cytometrical DNA profiles of RBT323 and RBT157 tumors
a: RBT323, first passage, b: RBT323, passage #2
c: RBT323, passage #14, d: RBT157, original tumor

Chromosomal Analysis

For cytogenetic studies the tumors were transplanted in female rats, the existence of the Y-chromosome could serve, therefore, as marker for karyotyping of tumor cells. Four RBT323 tumors and two RBT157 tumors were studied (Table II). All six tumors had a near diploid chromosome number with numerical and structural aberrations. RBT157 passage 2 had 4p+, 11p+, and a marker chromosome (M1) consistently. These structurally aberrations were conserved in RBT157 passage 4. All the RBT323 tumors had t(?::1q22->1qter) consistently. RBT323 pass. 4 and 7 had t(?::1p11->q54) and del(2) (q43) which were not observed in RBT323 pass 1 nor 2, resulting in structural abnormalities involving the short arm of both chromosome 1 in RBT323 pass. 4 and 7. There were no common structurally abnormal chromosomes between RBT157 and RBT323, while monosomies of 5 and 20 were observed consistently in RBT157 and RBT323.

Table II

	Modal chromosomal number	Range[a]	Specific Chromosomal Aberration	
			Numerical	Structural
RBT157 p2	41	39-42	+4,-5,-12,-12[a],-20	3p+,4p+,11p+,M1[c], 2 mars
RBT157 p4	42	41-43	+3,+4,-5,-12,-20	iso(3q),4p+,11p+,M1[c], 1-3 mars
RBT323 p1	42	41-43	-5,-8,-15,-19,-20	t(? 1q22->1qter), 4 6 mars
RBT323 p2	41	39-42	-5,-8,-13,-15,-19,-20	t(? 1q22->1qter), 3-6 mars
RBT323 p4	43	40-44	Y,-5,-8,-10,-19,-20	t(? 1q22->1qter), t(? 1p11->1q54) del(2)(q43),7mars
RBT323 p7	40	40	-Y,-5,-8,-10,-19,-20	t(? 1q22->1qter), t(? 1p11->1q54) del(2)(q43),8p+,5mars

[a] Range of chromosomal number in which 85% of counts were observed
[b] Specific changes underlined are not consistent Other changes are observed consistently (>80%)
[c] M1 is distinguished from the other markers, but origin of M1 is unknown

Table II Cytogenetic analyses of rat bladder tumor lines

In an autopsy series on 300 ACI rats we found five TCC. Two appeared to be serially transplantable (RBT 323 and- 157). Upon transplantation tumor biological characteristics changed. The tumor doubling time of the RBT323 tumor increased from 13 to 3.5 days. The RBT157 tumor has a lower doubling time, which is stable at 8 days. The most important phenotypic change is the metastatic ability. The RBT323 tumor changed in the 5th passage from low metastatic, less than 5% of the animals have metastases, to high metastatic ability, more than 90% of the animals show lung metastases and occasionally lymphnode metastases. This way of metastasizing suggests a haematogenic pathway. The metastatic ability of the RBT157 tumor changed in the 4th passage from low to intermediate, about 50% of the animals have microscopic lungmetastases. We sought to identify markers that are associated with the metastatic phenotype of the rat bladder tumors.

Light microscopy showed an invasive grade II TCC in the initial passages of the RBT323 tumor, which changed in the 3rd passage to grade III. The histological findings, therefore, do not correlate with the phenotypic changes. Even more, the histology of RBT 157 remained essentially unchanged, despite its progression to a moderate metastatic phenotype.

Electron microscopically superficial cells of the mammalian bladder are typically lined by an asymmetrical unit membrane (AUM). Within these cells numerous oblong elliptical vesicles derived from the Golgi area are found (38-40). The fact that we could demonstrate these vesicles, although in an aberrant form, stresses the urothelial origin of the bladder tumors. Some cells in the intermediate layer contain electron-dense membrane-bound granules, resembling (neuro)-endocrine granules, these cells have been described in human TCC also (41,42).

Intermediate filament proteins are expressed in a tissue type specific fashion and are therefore useful in tumor diagnosis (43). RBT 323 and-157 express cytokeratins and can therefore be considered of epithelial origin. So far 20 different cytokeratins have been characterized and their expression pattern is characteristic for the differentiation stage (25,26,44). Transitional cell epithelium typically shows expression of CK 5,7,8,18 and 19 (45,46). The synthesis of CK is usually maintained during malignant transformation and can, therefore be exploited in specific tumor diagnosis. With the panel of monoclonal antibodies used in this study we could not detect changes in the ratio RGE 53 positive cells/ keratin positive cells: enrichment of RGE53 positive cells which marks high grade human TCC was not evident in this model system (31). Since, this can be due to an "epitope" masking effect, it may in fact not be actual in rats. The basal cells of the RBT323 tumors, however, were not detected by RCK102 and the number of positive cells with RCK105 was less, when compared to the normal bladder and the RBT157 tumor, which were both completely positive with both antibodies. Currently studies with an extended panel of

monoclonal antibodies to greater number of CK are ongoing. Differences in expression patterns of CK 13 and 14 also have been suggested to be involved in bladder tumor progression (46). It should be noted that RKSE 60, recognizing keratin 10, which is found in keratinizing epithelium, was negative. This indicates that no squamous cell carcinoma components are represented in these tumors (29).

Clearly, the progression of tumor lines to a more aggressive phenotype (ie metastatic) is not marked by obvious histological changes. In an attempt to define the genetic changes associated with tumor progression, DNA content analysis by Flow Cytometry is usually helpful to establish gross genetic instability (47,48). The presence of an aneuploid cell population is considered a bad prognostic indicator, with limited usefulness for prognosis, since also diploid aggressive tumors are known to exist. We found that in the initial passages of RBT323, an aneuploid peak is present, and indeed this tumor line is the more aggressive one. In subsequent transplant generations, however, the aneuploid peak was not found, indicating that the genetic basis for the progression was expressed in the diploid tumor cell subpopulation. Aneuploidy, evident in the first transplant generations, may therefore have been an utterance of genetic instability but not per se being the subpopulation that progressed. This phenomenon may in fact explain the limited value of DNA measurements. The fact that we established a cell line from the first passage from this tumor line that was aneuploid, and non-tumorigenic (data not shown) further underlines the importance of the diploid cell population for tumor progression. Tumor line RBT157 was peridiploid in the first passage and remained so for the subsequent transplant generations.

Evidently, the progression to a metastatic phenotype is not associated with gross genetic changes. Cytogenetic studies, were therefore performed to establish whether more subtle genetic changes occurred during the progression of RBT323 and-157. Both the loss of genetic information, as well as, gain of function provoked by structural and/or numerical chromosomal changes are thought to be important. These analyses showed that both tumors were peridiploid with only few marker chromosomes. Moreover, in subsequent passages of each individual tumor line an accumulation of marker chromosomes was found, rather than selection for a subpopulation with a specific karyotype. In both rat tumors loss of chromosomes is found and maintained during tumor progression. One copy of chromosome 5,12 and 20 is consistently lost in the RBT157 tumor, likewise one copy of chromosome 5,8,19 and 20 is always absent in RBT323. Hence, chromosomes 5 and 20 are both lost in the independently arisen tumors, and this loss is maintained in all passages. No common structural changes, however, were found in the RBT323 and -157 series.

Thus we have characterized a panel of tumor lines derived from two independent superficial bladder tumors that represent the progression of bladder cancer. The progression is not characterized by gross phenotypic changes, yet is

associated with the accumulation of specific genetic changes. It is tempting to speculate that the model represents the more aggressive subgroup of superficial transitional cell carcinoma that progress to metastatic disease. We, therefore, think, that an important model system has been established that may be helpful in the identification of specific molecular steps associated with the progression of bladder cancer

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CHAPTER VIII

INHIBITION OF RAT BLADDER TUMOR (RBT323) GROWTH BY TUMOR NECROSIS FACTOR ALPHA AND INTERFERON-GAMMA IN VIVO

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ABSTRACT

The antiproliferative activities of recombinant rat gamma-interferon and recombinant human tumor necrosis factor alpha were evaluated in a new rat bladder tumor model, RBT323. The RBT323 is a spontaneously arisen, serially transplantable, bladder tumor in ACI rats. Histologically and ultrastructurally this tumor is a grade 2-3 transitional tumor without squamous cells. The RBT323 appears to be non-immunogenic in tumor excision and rechallenge experiments.

The tumor was transplanted subcutaneously and the drugs were administered peritumorally. Gamma-interferon and tumor necrosis factor monotherapy starting two days after tumor implantation showed dose-dependent growth inhibiting effects. Combination treatment with gamma-interferon and the highest dose of tumor necrosis factor resulted in synergistic antiproliferative effects. After cessation of the combination treatment all tumors resumed growth, initially with a low doubling time, but finally with their original growth rate. Gamma-interferon treatment initiated at a tumor volume of 0.2-0.5 cm^3 had no antitumor effects, whereas treatment with 100 μg tumor necrosis factor per rat inhibited tumor growth significantly. Different combinations of the cytokines had synergistic effects against established tumors.

These studies demonstrate antitumor effects of gamma-interferon and tumor necrosis factor in a rat bladder tumor model system. Combination therapy has synergistic effects and prolongs the time to tumor recurrence.

INTRODUCTION

In 1990 approximately 49,000 new cases of bladder cancer were diagnosed in the USA, of which 75 to 80% present as superficial tumors[1,2]. Bladder cancer, therefore, is the fourth most prevalent malignant disease among male and the ninth among female patients in the United States. It is now well established that 30 percent of the solitary papillary tumors, and more than 70 per cent of multiple tumors will recur after initial treatment[2,3]. 5 to 30 percent of all patients exhibit tumor progression to more invasive stages and eventually metastases[2,3]. The current therapy for patients presenting with recurrent superficial bladder tumors is endoscopic resection followed by adjuvant intravesical chemo- or immunotherapy, with e.g. thiotepa, doxorubicin, mitomycin C, or Bacillus Calmette Guerin (BCG). Although properly selected patients benefit from intravesical therapy, these agents do not consistently eradicate existing disease or prevent tumor recurrences[4]. Furthermore, each of the agents has been associated with local and/or systemic toxicities[4].

The mechanism of action of intravesical immunotherapy with BCG is largely unknown. Histopathological examinations before and after BCG application revealed an increase in cellular infiltrate in the bladder wall[5]. Another indication of the involvement of the immune system in the antitumor effects of BCG is the elevation of cytokines such as Interleukin-1 and -2, and Tumor Necrosis Factor (TNF) in the urine of BCG-treated patients. The local concentrations of TNF should be high enough to provide an antitumor effect[6].

TNF is a cytokine that can mediate the necrosis of subcutaneously implanted tumors. The neovasculature of transplanted and spontaneous tumors in mice is specifically affected by a single dose of TNF (5-20 μg/mouse), but there is no effect on the vascularization of normal tissue transplants[7]. TNF is synthesized by various activated phagocytic and non-phagocytic cells, including macrophages and monocytes, and has direct and indirect anticellular effects, mediated via the host immune system[8]. Growth of various tumors, e.g. renal and breast tumors, can be inhibited in animal model systems, although the antitumor effects against experimental bladder tumors are limited[9-14]. Recently, clinical trials with intravesical TNF instillations have been initiated[15].

Several preclinical and clinical studies have suggested that another cytokine, Interferon (IFN), can inhibit bladder tumor growth[16-18]. The IFNs are a family of naturally occurring glycoproteins, which share antiviral, immunomodulatory, and antiproliferative effects. Three major classes of IFNs (alpha, beta, and gamma) are defined on the basis of chemical, antigenic, and biological differences[19]. IFNs exhibit direct antitumor effects and can augment the effectiveness of immune effector cells, such as cytotoxic T-cells, natural killer cells, and monocytes. Moreover, the

expression of surface antigens, including HLA class I and II antigens on both tumor and effector cells and several oncogenes can be regulated by IFNs[19,20].

Combinations of cytokines can have synergistic antitumor effects. In previous studies we showed synergistic antiproliferative effects of gamma-IFN and TNF in a rat renal cell carcinoma model system[9]. In this study we report the antitumor effects of rat-gamma-IFN and human TNF in mono- and combination therapy in a new rat bladder tumor model system. Due to the species specificity of IFN we had to use the rat IFN[9]. In contrast to IFN, TNF has little species-specificity[9].

The RBT323 tumor is a spontaneously arisen bladder tumor in ACI rats[21] (manuscript submitted). Histologically and ultrastructurally it is a grade 2-3 transitional cell tumor with no squamous cells. Intermediate Filament Protein expression pattern analysis also revealed no squamous cell characteristics, i.e. expression of Cytokeratin 10 was not detected. Tumor cells were positive for Cytokeratins 7 and 18, which are specific for transitional cells.

MATERIALS AND METHODS

Animals

Adult male ACI/SegHSD rats (Harlan Sprague, Inc., Indianapolis, IN) were kept 3 rats per cage with a regimen of 12 hours light per day. The animals were allowed access to RMH-TM food (Hope Farms BV., Woerden, The Netherlands) and water ad libitum.

Tumor Line

For the experiments, transplant generation 8 to 13 of the RBT323 tumor were excised from a tumor-bearing animal, and after removal of normal and necrotic tissue, the tumor tissue was cut into pieces of 20 mg (18-23 mg). The precise weight of each tumor piece was measured on preweighed sterile gauzes on a laboratory scale (Mettler). An 0.5 cm incision was made in the right flank of recipient animals, and after separating the subcutaneous tissue by blunt dissection, the tumor fragment was placed subcutaneously (s.c.). The incision was closed using skin clips (7.5 x 1.75 mm, Aesculap, Germany). In this way a tumor take of more than 95% was achieved.

To test the immunogenicity of the rat bladder tumor, RBT323 tumors of passage 6 were completely removed together with the overlying skin and underlying subcutaneous tissues. The skin defect was closed with skin clips. Immediately after removal of this tumor, a tumor piece of 20 mg was implanted s.c. in the contralateral, left, flank.

Drugs

Recombinant human TNF alpha was kindly provided by Boehringer Ingelheim (Alkmaar, The Netherlands). The specific activity of TNF determined in the presence of Actinomycin D was 6 x 10^7 Units/mg protein as determined in the L-929 cytotoxicity assay. The purity was >99% as determined by SDS polyacrylamide gel electrophoresis and it contained less than 1.0 ng endotoxin per mg protein based on the limulus amoebocyte assay. The original TNF solution was diluted with RPMI 1640 culture medium (Gibco, Paisley, UK) to a final concentration of 2, 20, or 200 μg per ml, divided in aliquots and stored at -20^0 C.

Recombinant rat gamma-IFN was produced by a chinese hamster ovary (CHO) cell line transformed with a plasmid carrying the chromosomal gene for rat gamma-IFN and purified as described previously[22]. The cytopathic effect reduction assay was used for estimating gamma-IFN activity. Ratec cells (a continuous rat embryonic cell line) were incubated with gamma-IFN for 18 hours before they were challenged with vesicular stomatitis virus to determine the cytopathic effect. Because no internationally accepted reference standard for rat interferon is available, all titers were determined by comparison with a laboratory standard preparation of gamma-IFN (1 mg pure rat gamma-IFN corresponds to 7 x 10^6 Units). After purification by monoclonal antibody affinity chromatography gamma-IFN was freeze-dried for long-term storage. It was reconstituted with Milli-Q to a final concentration of 16,000 or 160,000 Units per ml, divided in aliquots and stored at -80^0 C until use.

Treatments and monitoring

Gamma-IFN and TNF were administered s.c., peritumorally, 3 and 5 times per week respectively, in a 0.5 ml injection volume. Phosphate buffered saline (PBS) was used for the control group. Treatment started two days after tumor implantation or at a tumor volume of 0.2-0.5 cm^3 and was continued until the tumors in the control group reached a volume of 5 cm^3. Tumor growth was followed by measurements of tumor size according to the method of Janik et al.[23] by the same measurer throughout each experiment. Three times per week length (l), width (w) and height (h) were measured using a slide caliper. No correction was made for skin thickness. The tumor volume (V) was calculated from the formula

$$V = 0.5236 \times l \times w \times h.$$

Statistical analysis

For each rat, the tumor volume was estimated 5 weeks after tumor implantation, i.e. time when the control group reaches the volume of 5 cm^3, by linear regression on the logarithms of the tumor volumes throughout this period. Two-sided t-tests were used to compare the final volumes of the treatment versus the control groups and to test for additive effects in the combination treatments. $P < 0.05$ is considered as statistically significant. 6 animals were included in each

group. The effect of treatment A is defined as $E_A = V_{control}/V_A$ and the effect of treatment B as $E_B = V_{control}/V_B$, where V_A, V_B, and $V_{control}$ are the estimates for the tumor volumes of treatment A, B or control group respectively. Additivity is defined[9] as $E_{A+B} = E_A \times E_B$ (i.e. $\log E_{A+B} = \log E_A + \log E_B$). A combination of two treatments is considered synergistic when the effect of the combination (E_{A+B}) is significantly greater than the product of the effects of the separate treatments.

RESULTS

To study the antitumor effects of human recombinant TNF-alpha and recombinant rat-IFN-gamma against bladder cancer, we have used a new, spontaneously arisen rat bladder tumor model. According to Hewitt, tumor models representative for human tumors should be spontaneously arisen and non-immunogenic[24]. We, therefore, first tested the immunogenicity of the tumor line.

Immunogenicity of RBT323 tumor line

After complete removal of RBT323 tumors with a volume of 5 cm^3, and subsequent reimplantation of a tumor piece in the contralateral flank of 8 rats, a tumor started to grow with the same growth rate and no lag-phase compared to tumors implanted in naive rats, suggesting that the tumor is non-immunogenic.

Cytokine monotherapy

In previous studies with a rat renal cell carcinoma model system we evaluated the influence of the administration route on antitumor effects of cytokines[9]. Comparison of intravenous (i.v.), intraperitoneal (i.p.) and subcutaneous (s.c.) routes showed that i.p. and i.v. injected cytokines were less effective than s.c. injections. We therefore administered gamma-IFN and TNF s.c., peritumorally.

Figures 1A and 1B depict the results of different dosages of gamma-IFN and TNF monotherapy. Gamma-IFN and TNF were administered three and five times per week respectively. Treatment started 2 days after tumor implantation and was continued for 5 weeks, i.e. the time taken for the control group tumors to reach a volume of 5 cm^3. 80,000 U gamma-IFN had a stronger antiproliferative effect than 8,000 U, suggesting a dose-dependent response (fig. 1A). Treatment with TNF also showed dose-dependent effects, 100 μg TNF per rat was more effective than the lower dose of 10 μg TNF (fig. 1B). The highest doses of both cytokines provoked a statistically significant growth inhibition.

Figure 1

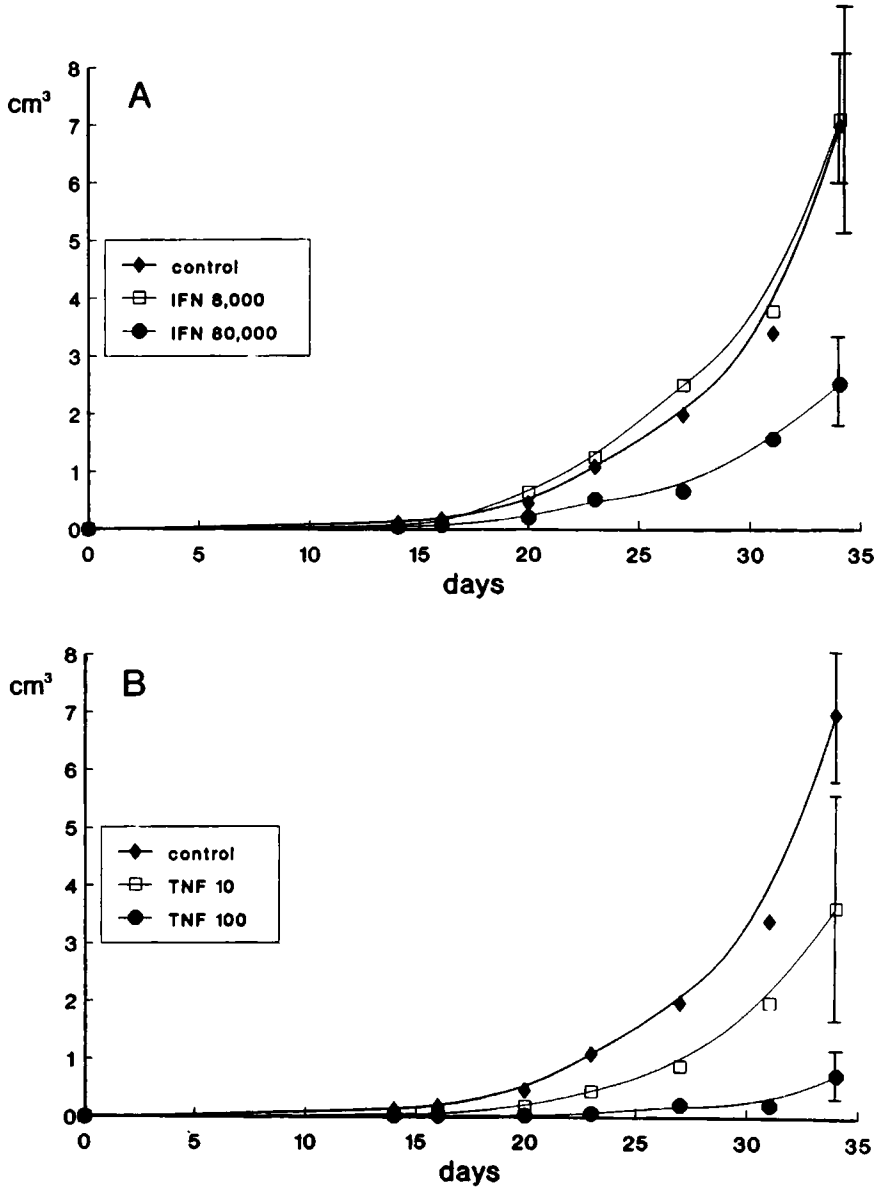

Figure 1: Effect of single agent therapy started 48 hours after tumor implantation. Bars indicate standard deviation. A: Gamma-IFN monotherapy administered s.c. peritumorally, 3 times per week; 8,000 U (n.s.) or 80,000 U ($p < 0.01$) per rat. B: TNF monotherapy, s.c. peritumorally, 5 times per week; 10 μg ($p = 0.02$) or 100 μg ($p = 0.0003$) per rat. n.s. = not significant

IFN and TNF combination therapy

As shown in figure 2 combinations of gamma-IFN and TNF can be more effective than their respective monotherapies. The combination of 8,000 U gamma-IFN and 10 μg TNF had no additive growth inhibiting effects, whereas a tenfold increase of the TNF dose produced a clear synergistic effect ($p = 0.008$). 2 out of six rats did not develop a tumor during the treatment period. However, after cessation of treatment all tumors started to grow again, finally with the same growth rate as the untreated control group.

Figure 2

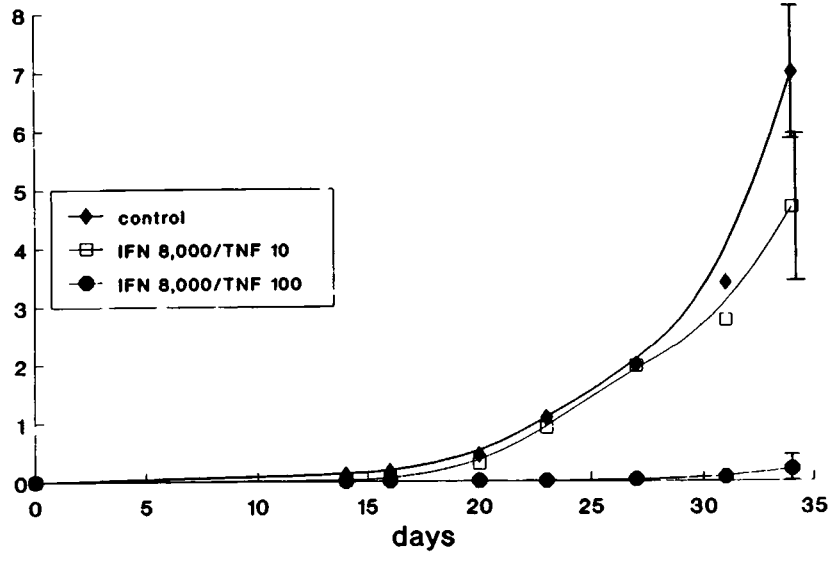

Figure 2. Combination therapy with gamma-IFN and TNF, 3 times and 5 times per week respectively. Treatment started 48 hours after tumor implantation. 8,000 U gamma-IFN and 10 μg TNF (n.s.) or 8,000 U IFN and 100 μg TNF ($p = 0.0007$) per rat.

In a separate experiment (fig. 3A) we tested the combination of 80,000 U gamma-IFN and 100 μg TNF. Rats receiving combination treatment lost 38 grams of their initial body weight during three weeks of treatment, whereas the rats in the other groups gained 15 grams in this period. For this reason, combination treatment was ceased earlier, i.e. time when control group reached a volume of 1 cc, and rats were evaluated for tumor recurrence. In the rats treated with the combination no tumors developed in the three weeks treatment period, whereas tumor growth was observed in the other three groups. Thus a synergistic antitumor effect of the combination was found ($p = 0.01$). At day 42 after tumor implantation the gamma-IFN/TNF treated rats were at their initial body weight. However, tumors were now also found in these rats, initially growing with a low doubling time, but the tumors

Figure 3

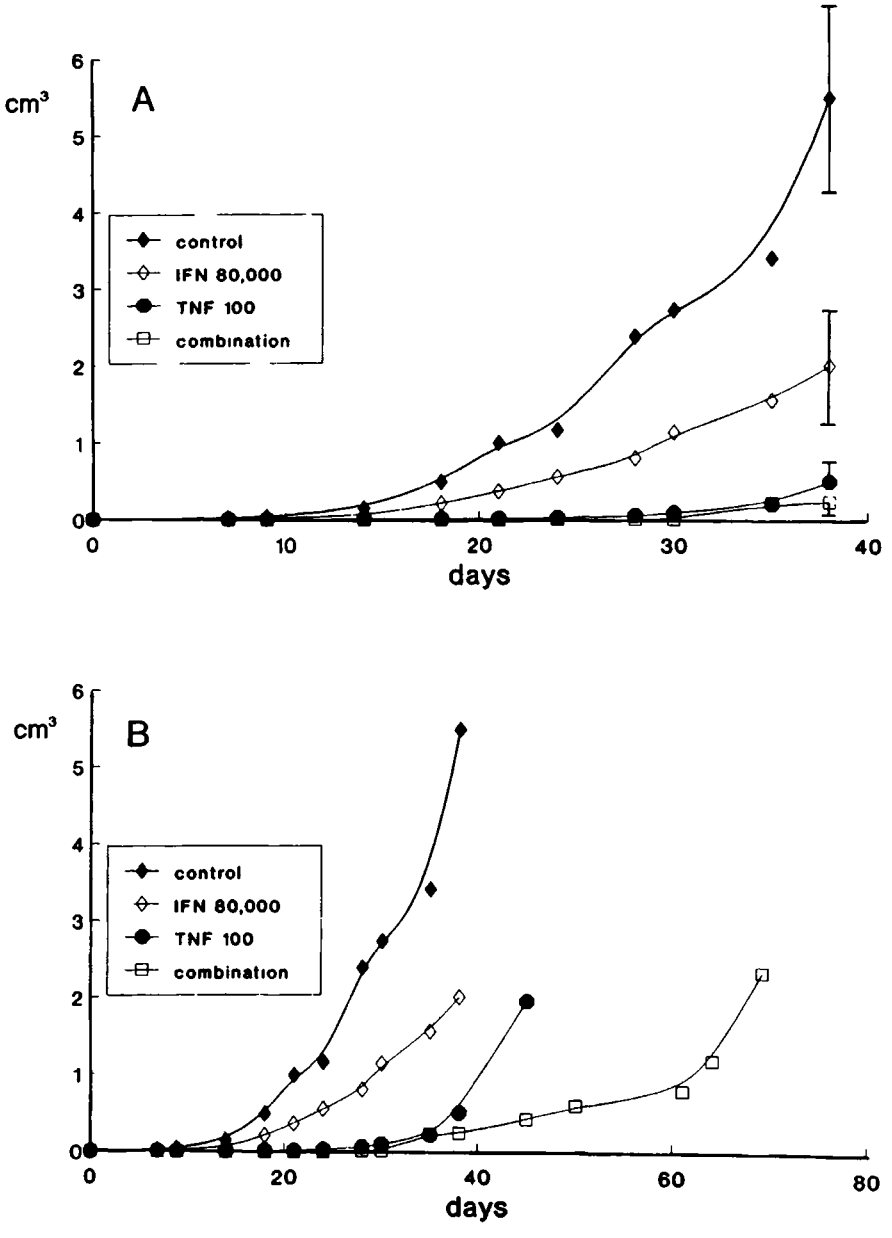

Figure 3. A: Effects of 80,000 U Gamma-IFN 3 times per week ($p < 0.01$) or 100 μg TNF 5 times per week ($p = 0.0001$) and their combination ($p < 0.0001$). Treatment started 48 hours after tumor implantation. B: Tumor growth after cessation of therapy at day 35 (monotherapies) or day 21 (combination treatment) after tumor implantation.

regained their original growth rate 64 days after tumor implantation (fig. 3B). The time to reach a tumor volume of 2 cm^3, however, was longer than in the monotherapies. The time to tumor recurrence was, therefore, prolonged by the combination treatment.

Influence of initial tumor volume

To assess the antiproliferative efficacy of gamma-IFN and TNF mono- and combination therapy on higher tumor volumes, therapy was started at 0.2-0.5 cm^3, i.e. 17-19 days after tumor implantation.

Gamma-IFN monotherapy at doses of 8,000 (fig. 4A) or 80,000 U per rat (fig. 5) did not significantly inhibit tumor growth. Also TNF monotherapy with the lowest dose of 10 μg per rat did not inhibit tumor growth. A tenfold higher dose, however, had significant antiproliferative effects (figs. 4A and 5).

The combination of 8,000 U gamma-IFN and 10 μg TNF revealed no potentiating effects (fig. 4B), but the combination of 8,000 U gamma-IFN and 100 μg TNF showed significant synergistic antiproliferative effects ($p = 0.006$). 2 out of six rats showed stabilization of tumor volume during the treatment period. Treatment with the combination of 80,000 U gamma-IFN and 100 μg TNF also had synergistic antitumor effects ($p = 0.04$, fig. 5). 2 tumors showed regression, 1 tumor showed stabilization, whereas the remaining three tumors had a very strong reduction of their volume doubling time. Interestingly, these rats did not show any sign of toxicity.

Rats receiving combination therapy which included 100 μg TNF, showed tumor growth after cessation of treatment with a growth pattern similar to that shown in fig. 3B. The initial growth rate of the tumors was low, but finally all tumors regained their original growth rate.

DISCUSSION

According to Hewitt the ideal tumor models to use for immunotherapeutical studies should be spontaneously arisen and non-immunogenic[24]. Since most carcinogen- or virus-induced tumors carry tumor-associated antigens on their cell surface, these tumors will be immunogenic[25]. Up to now most immunotherapeutical studies in experimental bladder cancer are carried out in the immunogenic murine FANFT-induced MBT-2 tumor model[11-13,16,26]. It is possible to elicit an immune reaction against MBT-2 tumor vaccines with whole tumor cells or cell extracts, but these mechanisms are not likely to contribute significantly to the host immune defense in human transitional cell carcinoma[27]. To gain further insight in the efficacy of immunotherapy with cytokines against bladder carcinoma it is necessary

Figure 4

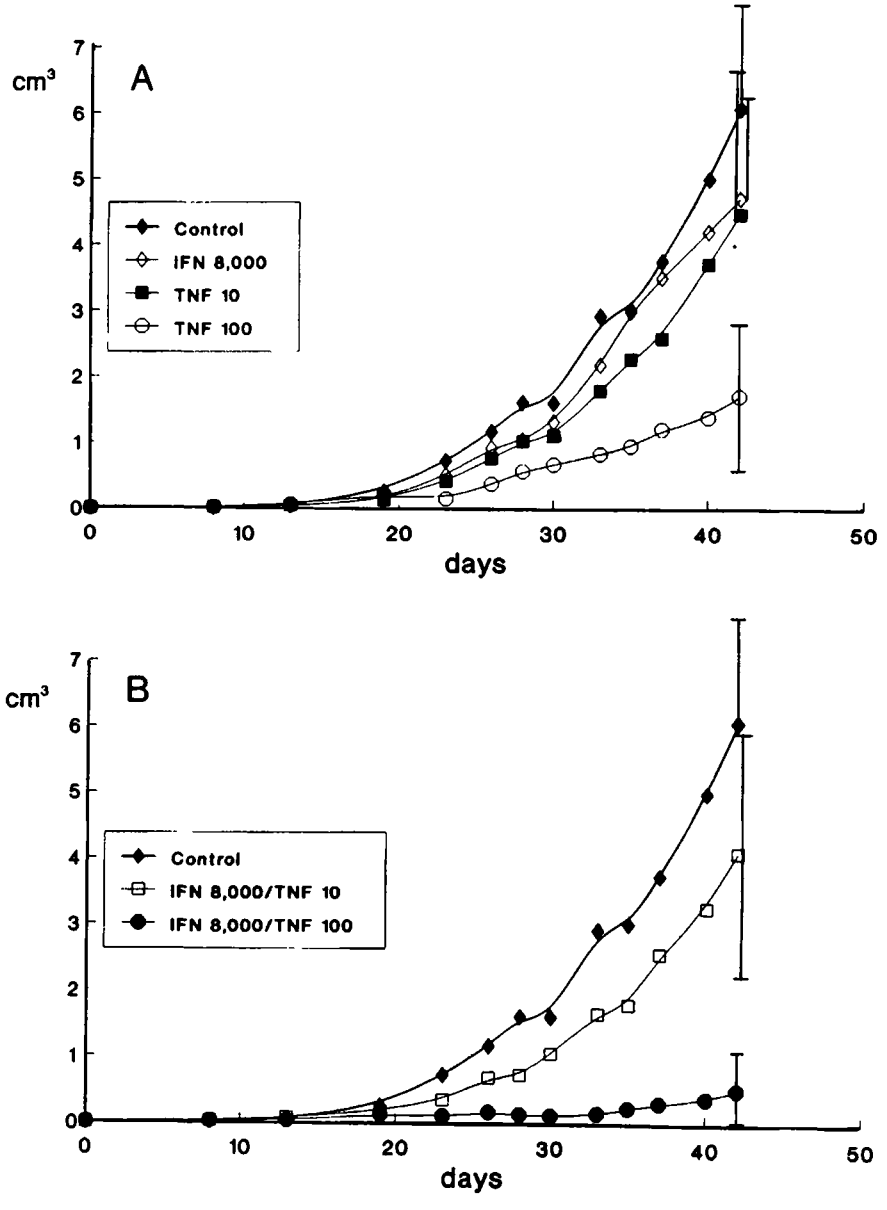

Figure 4. Start of treatment at tumor volume of 0.2-0.5 cm^3. Gamma-IFN 3 times and TNF 5 times per week, s.c., peritumorally. A: Monotherapies: Gamma-IFN 8,000 U (n.s.), TNF 10 μg (n.s.) or TNF 100 μg ($p = 0.0003$) per rat. B: Combination therapy with combination of 8,000 U Gamma-IFN and 10 μg TNF ($p < 0.05$) or 8,000 U Gamma-IFN and 100 μg TNF ($p = 0.0003$) per rat.

Figure 5

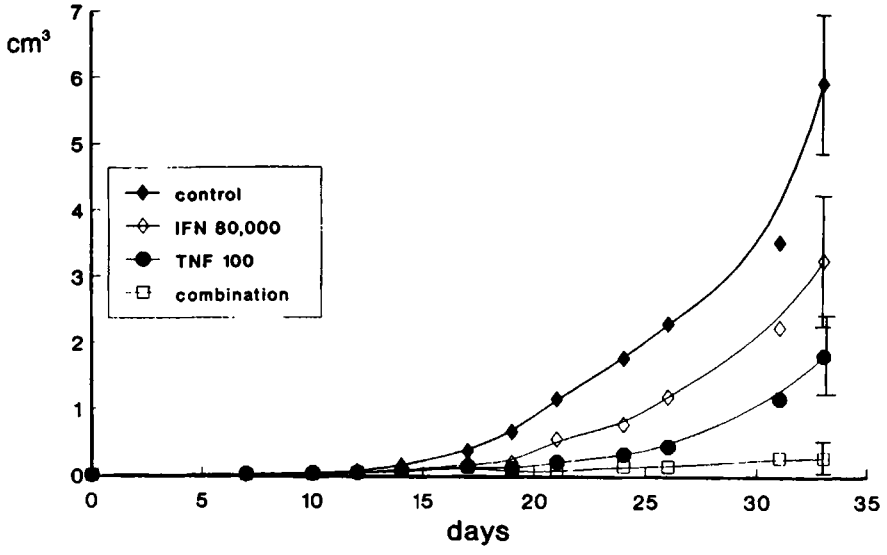

Figure 5. Effects of 80,000 U Gamma-IFN 3 times per week (p = 0.005) or 100 μg TNF 5 times per week (p = 0.0002) and their combination (p = 0.003). Treatment started at tumor volume of 0.2-0.5 cm^3.

to use more tumor models in different animals. We, therefore, examined the antitumor effects of rat-gamma-IFN and TNF in the spontaneously arisen and non-immunogenic rat bladder tumor model system, RBT323[21].

The antitumor effect of TNF has now been established in various animal model systems[7,9,10]. The activity against the MBT-2 tumor, however, is limited. Studies with TNF derived from supernatants of the endotoxin stimulated J774.1 macrophage cell line showed in vitro cytotoxicity, this crude TNF also significantly suppressed growth of subcutaneous tumors[12]. Recombinant TNF alone, or in combination with a low dose Actinomycin D had no cytotoxic effects in vitro, furthermore, intravesical treatment with 10 μg TNF did not reduce the outgrowth of bladder tumors in vivo[13]. In contrast to these results, Lee et al. observed a significant reduction in MBT-2 tumor weight with intravesical crude and recombinant TNF treatment in a dose-dependent fashion[11]. The tumor incidence was lower with crude TNF, which was not the case with the recombinant TNF. The differences in antitumor activity may be explained by admixture of other cytokines or endotoxins in the crude TNF preparations or by differences between human and murine TNF. In the KK-47 bladder tumor xenograft model, intravenously or intratumorally administered recombinant TNF did not inhibit the growth of subcutaneously

implanted tumors[14]. In our model we showed a dose-dependent tumor growth inhibition by TNF injected peritumorally, 5 times per week. TNF 10 μg per rat was not effective, whereas TNF 100 μg per rat induced clear antitumor effects, this dose also inhibited growth of established tumors (0.2-0.5 cm^3).

In vitro studies using human cell lines showed antiproliferative effects of all three IFN types[20]. Mouse IFN-alpha,beta inhibited the in vitro proliferation of MBT-2 cells[16], however, in vivo treatment with this IFN type was not effective. Combination treatment with Adriamycin or DFMO, did not increase the effects of IFN-alpha,beta in vivo[16]. In our studies start of rat gamma-IFN treatment 48 hours after RBT323 tumor implantation resulted in a dose-dependent antiproliferative effect, however, treatment initiated at a higher tumor volume was not effective. The same relation of IFN effectiveness to tumor mass was found for beta-IFN in the treatment of the RT4 bladder tumor xenograft in nude mice and in previous experiments in a rat renal tumor model system[9,28]. Multiple clinical trials have been carried out with different IFN-types. Horoscewicz reviewed nine clinical trials and found 41% objective responses among 100 patients with bladder cancer[17]. Furthermore, it is clear that the number of side effects is low[17,18].

Different combinations of cytokines can result in the enhancement of their antiproliferative effects, e.g. the combination of TNF and rat gamma-IFN has synergistic antitumor effects in breast and renal tumor models[9,10]. MBT-2 cells 4 hours pretreated with mouse-gamma-IFN were more sensitive to TNF in vitro[13]. However, 30,000 U mouse-gamma-IFN intravenously combined with 2.5 or 12.5 μg TNF intravesically, did not show additional tumor suppression of the MBT-2 tumor in the dose range tested[29]. In contrast to these results, combinations of both doses rat gamma-IFN and 100 μg TNF showed synergistic effects against low and high tumor volumes of the RBT323 tumor, the latter being more representative for the clinical situation.

In the experiments described we injected the drugs peritumorally, in order to get a direct contact of the drugs with the tumor, similar to intravesical drug administration. However, once the tumor becomes metastatic this local treatment is not effective anymore. Systemic administration of the doses used in our experiments will result in lower local drug levels and, therefore, lower antitumor effects. Due to the toxicity of TNF an unlimited increase of the administered dose is not possible. Small changes in the TNF molecule made it less toxic, this TNF-s has already shown to have antitumor effects in experimental bladder tumors[30]. Encapsulation of cytokines in liposomes also lowers their cytotoxic effects[31]. Specific targeting of cytokines to metastases would rise local drug levels with subsequent improved antitumor effects. A possibility could be the use of cytokines coupled to a monoclonal antibody directed to a bladder carcinoma cell surface-expressed antigen.

In conclusion: cytokine treatment with TNF and gamma-IFN monotherapy showed antitumor effects in the rat bladder tumor model system. Combinations of

the cytokines had synergistic effects, also against established tumors. Treatment of low tumor volumes with the combination of 80,000 U gamma-IFN and 100 μg TNF showed no tumor growth during the treatment and a clear growth delay after cessation of treatment. Unfortunately, finally all tumors started to grow with the original doubling time. More research is needed to investigate other cytokine combinations and to elucidate the mechanism of action of the cytokines. The RBT323 tumor can be instrumental for both purposes.

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SUMMARY AND PROSPECTS

Summary

In **chapter I, part 1**, an overview is given of the available animal model systems for renal, prostatic and bladder cancer. Generally, tumor models can be divided into four categories: drug induced tumors, virus induced tumors, spontaneous tumors and heterotransplanted human tumors in immune-deprived animals. The best tumor models for performing immunotherapeutical studies are the spontaneously arisen tumors. **Part 2** of the general introduction gives an overview of the current knowledge of interferon (IFN) and tumor necrosis factor (TNF) and their use in the treatment of urological cancers. The antiproliferative effects of the cytokines can be divided into direct effects on the tumor cells, and indirect effects, mediated by the immune system. Although a great number of studies have been performed to elucidate the mechanism of action of these cytokines, current knowledge is still fragmentary and many questions regarding the optimal doses, the best route of administration, the best cytokine combination and the intracellular effects of IFN and TNF remain hitherto unanswered.

IFN is the most widely used cytokine in immunotherapy of renal cell carcinoma (RCC). All preclinical studies using IFN and/or TNF are performed in cell lines or xenografts transplanted into nude mice. In 1987 we, therefore, started our studies with the syngeneic rat renal cell tumor model system, as described in **chapter II**. Pilot-studies showed that intraperitoneally or intravenously injected cytokines were less effective than subcutaneous (s.c.) injections. Therefore we injected IFN and TNF s.c., peritumorally in all in vivo experiments described in this thesis. Rat-gamma-interferon had dose-dependent antitumor effects, whereas TNF was only effective at the highest dose level. Different combinations of IFN and TNF had additive or synergistic effects. The combination of both highest doses completely inhibited tumor growth. The antitumor effects of the cytokines appeared to be dependent on the tumor volume at the start of therapy. Cytokine monotherapy could not inhibit growth of tumors with a volume of 0.2-0.5 cm^3. The combination of both highest doses significantly inhibited tumor growth, but did not provoke tumor regression. These studies clearly show that cytokine combination treatment is more effective than monotherapy. Furthermore, treatment should be started as soon as possible, i.e. at low tumor burden.

In **chapter III** we describe the antitumor effects of TNF and/or radiotherapy against rat renal cell tumor spheroids. TNF monotherapy had a dose-dependent growth inhibiting effect. Single-dose irradiation also had dose-dependent antitumor effects, although this was not a linear dose-dependency. In combination treatments, TNF was added 4 hours prior to irradiation. TNF did not induce a potentiation of

the lowest dose of radiation. However, the combinations with the higher doses induced additive or synergistic antitumor effects. This study, and studies done by others (1-4) indicate that the combination of TNF and radiotherapy may be useful in clinical cancer therapy.

The combination of cytokines with chemotherapeutical agent seems very promising (5). In vitro studies showed marked enhancement of the cytotoxicity of TNF in combination with chemotherapeutical agents directed against DNA-topoisomerase II (6). In vivo studies using RCC xenografts also showed synergism between VP16 and TNF (7,8). We have investigated the in vitro and in vivo antitumor effects of TNF and Adriamycin or VP16 (**chapter IV**). Adriamycin monotherapy was already very active in vitro, therefore only the lowest dose produced synergistic effects in combination with TNF. Different combinations of VP16 and TNF produced significant antitumor effects, but none of these combinations had synergistic effects. The highest dose of Adriamycin in vivo (1 mg/kg bodyweight) administered for three consecutive days every two weeks, had significant antitumor effects, and induced synergistic effects in combination with TNF. The same dose given for three consecutive days every three weeks showed neither significant, nor synergistic effects in combination with TNF. Similar to the studies presented in chapter II, TNF and Adriamycin monotherapy were not effective when treatment started at a tumor volume of 0.2-0.5 cm^3. But, TNF and Adriamycin combination therapy was not effective either. VP16 monotherapy did not induce significant antitumor effects, and VP16 in combination with TNF did not show substantial potentiation of the therapeutic efficacy. In conclusion: TNF and IFN-gamma combination therapy is more effective than the combination of TNF with chemotherapeutical agents directed against DNA-topoisomerase II in the syngeneic model system studied.

In **chapter V** we studied the antitumor effects of rat-gamma-IFN and TNF in the syngeneic Dunning R3327 rat prostatic tumor model system. In vitro studies with three different androgen-independently growing cell lines showed very limited antiproliferative effects of IFN and TNF mono- and combination therapy. For in vivo studies androgen-dependent and -independent tumor lines were used. Except for IFN monotherapy against the MatLyLu line, cytokine monotherapy was not effective. Combination therapy, however, had synergistic antitumor effects against all four cell lines tested. Survival studies showed a significant increase in survival of MatLyLu tumor-bearing rats. **Chapter VI** describes the in vivo antitumor effects of human IFN-alpha and -gamma and TNF against two androgen-independently growing prostatic tumor xenografts, PC3 and DU145. TNF 500 ng/gram bodyweight was the only monotherapy effective against both xenografts. Combinations of both IFNs were effective against the PC3 tumor, but not against the DU145 tumor.

Combinations of IFN-alpha and TNF were very effective against both xenografts. IFN-gamma and TNF combinations also showed significant antitumor effects against both lines. The data presented in chapter V and VI indicate that cytokines can have antiproliferative effects against hormone-dependent and -independent prostatic tumors. This may open a way to a new therapeutic modality in the treatment of prostatic cancer.

The number of spontaneous bladder tumor model systems is very limited. In **chapter VII** we present the characterization of two new transplantable transitional cell carcinoma (TCC) lines in ACI rats, rat bladder tumor (RBT) 323 and RBT157. The metastatic ability of the RBT323 tumor changed from low (<5% of the rats have lungmetastases) to high (more than 90%) in the 5th passage. In the 4th transplant generation the RBT157 tumor became metastatic to the lungs in about 50% of the animals. Histological, electronmicroscopical and immunohistochemical studies showed that the tumors have the typical characteristics of TCC. The progression of the tumors to a metastatic phenotype, however, was not associated with a specific change in morphology. Cytogenetic analysis of different passages showed that both tumors are peridiploid with few marker chromosomes in the initial passages. In subsequent passages an accumulation of marker chromosomes was found. Furthermore, in both tumors a loss of chromosomes was found which maintained during tumor progression. Thus, we have characterized a panel of tumor lines derived from two independently arisen superficial bladder tumors that represent the progression of bladder cancer and that may be helpful in the identification of specific molecular steps associated with the progression of bladder cancer.

Chapter VIII presents the data obtained with rat-gamma-IFN and TNF treatment of the RBT323 tumor. IFN and TNF monotherapy had dose-dependent growth inhibiting effects. Combination therapy resulted in synergistic effects. IFN had no antitumor effects against tumors of 0.2-0.5 cm^3, whereas TNF monotherapy inhibited tumor growth significantly. Different combinations of both cytokines had synergistic effects against established tumors.

Prospects

In the studies presented here we have administered the cytokines subcutaneously, peritumorally. In clinical trials, however, cytokines will be administered subcutaneously, intramuscularly or intravenously, inevitably resulting in lower local cytokine levels. Bartsch et al. tried to overcome this problem by intralesional application of TNF (9). In five out of 14 patients with different advanced solid tumors local tumor regression occurred. The duration of the response was short, suggesting a rapid development of resistance to TNF. Another route of administration resulting in high local drug levels is selective direct arterial infusion of TNF (10).

Clinical trials have shown that cytokines, especially TNF, can have major dose-limiting side-effects, such as hypotension. Other common side-effects include fever, rigors, nausea and vomiting at relatively low doses. Different approaches can overcome this toxicity; a single injection of indomethacin or ibuprofen 2 hours before TNF administration or bismuth subnitrate (50 mg/kg/day) 5 days before TNF administration, reduces the toxic effects of TNF, without preventing its anti-neoplastic actions in animal model systems (11,12). In clinical trials, Fosså et al. and Creagan et al. showed similar results with the combination of IFN-alpha with corticosteroids or aspirin (13,14). Another possibility to overcome side-effects is the use of liposome-encapsulated cytokines (15,16). Gatanaga et al. constructed novel TNF molecules (TNF-s) and showed reduced toxicity of these molecules without loss of antitumor activity (17).

Clinical experience with cytokines indicate that a subset of patients is sensitive to this therapy. However, it has been impossible to predict which patients will respond to cytokine therapy. In an interesting paper, Nanus et al. found that the expression of a kidney-associated differentiation glycoprotein, gp160, correlated with resistance to IFN-alpha therapy, and, conversely, that lack of expression correlated with sensitivity to IFN-alpha in vitro and in vivo (18). It has been shown that molecules of the major histocompatibility complex (MHC) can be upregulated by cytokines. The inducibility of both MHC class I and II antigens appeared to be a predictive marker for response to IFN and TNF therapy in nude mice (19). However, class I and II expression of untreated tumors were not related to the antitumor effects of both cytokines (19). A retrospective study is ongoing in which we test the hypothesis whether the pretreatment MHC expression of primary and metastatic RCC specimens is related to the effectivity of IFN-alpha and -gamma combination treatment.

More studies are needed to elucidate the mechanism of action of cytokines. In the rat renal cell model system we are currently investigating the lymphocyte-infiltration in tumors before and after cytokine treatment. Preliminary data indicate an increase of macrophages after TNF treatment. In all rat models used in this thesis, human TNF was not toxic to rats, although TNF was given in high doses.

This phenomenon could be due to the development of TNF-neutralizing antibodies in rats. However, in a preliminary study with serum of 12 rats, performed at the TNO REP-institutes, Rijswijk, no TNF-neutralizing antibodies were found.

In the near future, the use of recombinant DNA-technology will provide new cytokines, such as IFN-alphas (20) and hybrid combination molecules, such as IFN-gamma plus TNF-beta (21). For testing of new immunological approaches, the availability of a valid pre-clinical screen is very important (22). The animal models used in this thesis seem to provide such a preclinical screen. Furthermore, new immunotherapeutical modalities, such as monoclonal antibodies (23) and new cytokine combinations can be tested in these models.

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Samenvatting

In **hoofdstuk I, deel 1** wordt een overzicht gegeven van de beschikbare diermodellen voor nier-, prostaat- en blaaskanker. In het algemeen kunnen deze modellen worden ingedeeld in 4 categorieën: tumoren geïnduceerd door carcinogene stoffen, viraal-geïnduceerde tumoren, spontaan ontstane tumoren en humane tumoren getransplanteerd in dieren met een gestoorde afweer (heterotransplantatie). De beste tumormodellen voor het uitvoeren van immunotherapeutische studies zijn de spontaan ontstane tumoren. **Deel 2** van de algemene introductie geeft een overzicht van de huidige kennis betreffende interferon (IFN) en tumor necrosis factor (TNF) en het gebruik van deze cytokines in de behandeling van urologische tumoren. De antiproliferatieve effecten kunnen verdeeld worden in effecten direct aangrijpend op de tumorcellen en indirecte effecten, gemedieerd door het immuunsysteem. Hoewel er reeds een groot aantal studies zijn uitgevoerd om het werkingsmechanisme van deze cytokines te bestuderen, blijft de huidige kennis fragmentarisch. Vele vragen betreffende de optimale dosering, de beste toedieningsroute, de beste combinatietherapie en de intracellulaire effecten van IFN en TNF blijven tot nu toe onbeantwoord.

IFN is de meest toegepaste cytokine bij de immunotherapie van niertumoren. Alle preklinische studies met IFN en TNF hebben gebruik gemaakt van cellijnen of xenografts. Daarom zijn wij in 1987 gestart met onze studies in het ratten-niertumormodelsysteem, zoals beschreven in **hoofdstuk II**. Pilot-studies lieten zien dat intraperitoneaal of intraveneus toegediende cytokines minder effectief waren dan subcutane injecties. Daarom zijn in alle hieropvolgende experimenten IFN en TNF subcutaan, peritumoraal gespoten. Rat-gamma-IFN had dosis-afhankelijke antitumor effecten, terwijl TNF alleen effectief was in de hoogste dosering. Verschillende combinaties van IFN en TNF hadden additieve of synergistische effecten. De combinatie van de beide hoogste doseringen resulteerde in complete groeiremming van de tumoren. De antitumor effecten bleken afhankelijk te zijn van het tumorvolume bij aanvang van de therapie. Cytokine monotherapie kon niet de groei remmen van tumoren met een volume van 0,2-0,5 cm^3. De combinatie van beide hoogste doseringen gaf een significante groeiremming, maar veroorzaakte geen tumorregressie. Deze studies tonen duidelijk aan dat cytokine combinatietherapie effectiever is dan monotherapie. Bovendien moet de behandeling zo snel mogelijk gestart worden, dat wil zeggen, bij een klein tumorvolume.

In **hoofdstuk III** beschrijven we de antitumor effecten van TNF en/of radiotherapie tegen ratten-niertumor spheroiden. TNF monotherapie had een

dosisafhankelijk groeiremmend effect. Eénmalige bestraling had ook een dosisafhankelijk effect, hoewel dit geen lineaire dosisafhankelijkheid was. Bij de combinatiebehandeling werd TNF vier uur voorafgaande aan de bestraling toegediend. TNF veroorzaakte geen versterking van het effect van de laagste bestralingsdosis. Echter de combinaties met de hogere doses induceerden additieve of synergistische effecten. Onze en andere studies (1-4) laten zien dat de combinatie van TNF en radiotherapie bruikbaar kan zijn in de behandeling van kanker.

De combinatie van cytokines met chemotherapeutica lijkt veelbelovend (5). In vitro studies lieten een duidelijke versterking van de cytotoxiciteit van TNF zien in combinatie met chemotherapeutica gericht tegen DNA-topoisomerase-II (6). In vivo studies met niertumor xenografts toonden ook synergisme tussen VP16 en TNF (7,8). Wij hebben de antitumor effecten van TNF en Adriamycine of VP16 in vitro en in vivo onderzocht in het syngene ratten-niertumormodelsysteem (**hoofdstuk IV**). Adriamycine monotherapie bleek in vitro reeds zeer effectief te zijn en daarom resulteerde alleen de combinatie van de laagste dosis met TNF in een synergistische remming van de kolonievorming. Verschillende combinaties van VP16 en TNF resulteerden in significante groeiremming in vitro, maar geen van deze combinaties vertoonde synergisme. De hoogste dosering van Adriamycine (1 mg/kg lichaamsgewicht), toegediend op drie opeenvolgende dagen iedere twee weken, gaf significante antitumor effecten en induceerde in combinatie met TNF synergistische effecten. Dezelfde dosis gegeven op drie opeenvolgende dagen iedere drie weken toonde noch significante noch synergistische effecten in combinatie met TNF. TNF en Adriamycine monotherapie waren niet effectief tegen tumoren van 0,2-0,5 cm^3, dit is in overeenstemming met de studies gepresenteerd in hoofdstuk II. Echter TNF en Adriamycine combinatietherapie was ook niet werkzaam op gevestigde tumoren. VP16 monotherapie induceerde geen significante antitumor effecten en bovendien resulteerde VP16 in combinatie met TNF niet in een wezenlijke versterking van effectiviteit. Concluderend blijkt TNF in combinatie met IFN-gamma effectiever te zijn in het syngene ratten-niertumormodel dan TNF in combinatie met chemotherapeutica gericht tegen DNA-topoisomerase-II.

In **hoofdstuk V** onderzochten we de antitumor effecten van rat-gamma-IFN en TNF in het syngene Dunning R3327 ratten-prostaattumor modelsysteem. In vitro studies met drie androgeen-onafhankelijk groeiende cellijnen lieten zeer beperkte antiproliferatieve effecten van IFN en TNF mono- en combinatietherapie zien. Voor de in vivo studies werden androgeen-afhankelijke en -onafhankelijke tumorlijnen gebruikt. Cytokine monotherapie was niet werkzaam, met uitzondering van IFN tegen de MatLyLu lijn. Combinatietherapie had echter synergistische effecten op alle vier geteste tumorlijnen. Overlevingstudies toonden een significante levensverlenging aan van de ratten met de MatLyLu-tumor. **Hoofdstuk VI** beschrijft

in vivo experimenten met humaan IFN-alpha en -gamma en TNF tegen twee androgeen-onafhankelijk groeiende prostaattumor xenografts, PC3 en DU145. TNF in een dosering van 500 ng/gram lichaamsgewicht was de enige monotherapie die werkzaam was tegen beide xenografts. Combinaties van beide types IFN waren alleen effectief tegen de PC3 tumor, maar niet tegen de DU145 tumor. Combinaties van IFN-alpha en TNF waren zeer effectief tegen beide xenografts. Ook de combinatie van IFN-gamma en TNF resulteerde in significante antitumor effecten tegen beide tumorlijnen. De in hoofdstuk V en VI gepresenteerde gegevens laten zien dat cytokines antiproliferatieve effecten hebben tegen hormoon-afhankelijke en -onafhankelijke prostaattumoren. Dit kan misschien leiden tot een nieuwe therapievorm in de behandeling van prostaattumoren.

Het aantal spontaan ontstane blaastumor-modelsystemen is zeer beperkt. In **hoofdstuk VII** wordt de karakterisatie van twee nieuwe, transplanteerbare overgangscelcarcinoom (TCC) lijnen in ACI ratten, de ratten-blaastumor (RBT) 323 en RBT157, beschreven. De metastaserende capaciteit van de RBT323 tumor veranderde in de vijfde transplantatie generatie van laag (minder dan 5%) naar hoog (meer dan 90% van de ratten hebben longmetastasen). In de vierde passage veranderde de neiging tot metastasering van de RBT157 tumor van laag naar intermediair (longmetastasen in ongeveer 50% van de dieren). Histologisch, electronenmicroscopisch en immuunhistochemisch onderzoek toonde aan dat de tumoren de typische karakteristieken van TCC hebben. De progressie van de tumoren tot een metastaserend phenotype was echter niet geassocieerd met een specifieke verandering in de morfologische kenmerken. Cytogenetische analyse van verschillende passages toonde aan dat beide tumoren peridiploid zijn met weinig markerchromosomen in de eerste passages. In de daaropvolgende passages werd een accumulatie van markerchromosomen gevonden. Bovendien werd in beide tumoren een verlies van chromosomen gevonden, wat bleef bestaan gedurende verdere tumorprogressie. Hiermee hebben wij een panel van tumorlijnen gekarakteriseerd, afgeleid van twee onafhankelijk ontstane oppervlakkige blaastumoren, dat de tumorprogressie van blaaskanker representeert. Deze lijnen kunnen van nut zijn bij het onderzoek naar specifieke moleculaire veranderingen geassocieerd met de progressie van blaaskanker.

Hoofdstuk VIII beschrijft de experimenten met rat-gamma-IFN en/of TNF tegen de RBT323 tumor. IFN en TNF monotherapie hadden een dosisafhankelijk groeiremmend effect. Combinatietherapie resulteerde in synergistische effecten. IFN monotherapie had geen groeiremmend effect op tumoren van 0,2-0,5 cm^3, terwijl TNF monotherapie bij dit tumorvolume de groei wel significant remde. Verschillende combinaties van beide cytokines lieten synergistische antitumor effecten tegen gevestigde tumoren zien.

Toekomstverwachtingen

In de hier beschreven studies werden de cytokines subcutaan, rond de tumor gespoten. In klinische trials zullen de cytokines echter subcutaan, intramusculair of intraveneus toegediend worden, hetgeen onvermijdelijk resulteert in lagere lokale cytokine concentraties. Bartsch et al. probeerden dit probleem op te lossen door TNF intratumoraal toe te dienen (9). In vijf van de veertien patiënten met tumoren in een vergevorderd stadium trad lokale tumorregressie op. De duur van de respons was kort wat zou kunnen wijzen op een snel ontstaan van resistentie tegen TNF. Een andere mogelijkheid om hogere lokale cytokine concentraties te krijgen is selectieve intra-arteriële toediening (10).

Klinische trials hebben laten zien dat cytokines, met name TNF, belangrijke dosisbeperkende bijwerkingen kunnen hebben, zoals hypotensie. Andere veel voorkomende bijwerkingen die reeds bij lagere doseringen optreden zijn: koorts, krampen, misselijkheid en braken. Er zijn verschillende methoden om deze toxiciteit tegen te gaan; een eenmalige injectie van indomethacine of ibuprofen twee uur voor toediening of bismuth subnitrate (50 mg/kg/dag) 5 dagen voor toediening van TNF vermindert de toxische effecten van TNF in diermodellen zonder dat de antitumor werking teniet gedaan wordt (11,12). Fosså et al. and Creagan et al. lieten in klinische trials gelijksoortige effecten zien bij de combinatie van IFN-alpha met corticosteroiden of aspirine (13,14). Een andere manier om bijwerkingen te voorkomen is het gebruik van cytokine-bevattende liposomen (15,16). Gatanaga et al. construeerden nieuwe TNF-moleculen (TNF-s) en vonden een verminderde toxiciteit van deze moleculen zonder verlaging van de antitumor activiteit (17).

Klinische resultaten met cytokines laten zien dat een subgroep van de patiënten reageert op deze therapie. Het is echter tot nu toe onmogelijk te voorspellen welke patiënten zullen reageren. In een interessante studie beschrijven Nanus et al. dat de expressie van een nier-geassocieerd glycoproteïne, gp160, correleerde met resistentie tegen IFN-alpha therapie en omgekeerd, dat afwezigheid van gp160-expressie correleerde met gevoeligheid voor IFN-alpha in vitro en in vivo (18). Cytokines kunnen de expressie van moleculen van het major histocompatibiliteitscomplex (MHC) verhogen. De induceerbaarheid van MHC klasse I en II antigenen bleek een voorspellende waarde te hebben voor de respons op cytokine therapie in naakte muizen (19). Echter, klasse I en II expressie van onbehandelde tumoren was niet gerelateerd met antitumor effecten van beide cytokines (19). Momenteel is een retrospectieve klinische studie gaande waarin onderzocht wordt of de MHC expressie van tumoren bij aanvang van behandeling met IFN-alpha en -gamma gerelateerd is aan de effectiviteit van de behandeling en als marker met voorspellende waarde voor respons kan dienen.

Meer studies zijn nodig om het werkingsmechanisme van de cytokines te ontrafelen. In het ratten-niertumormodel wordt momenteel de lymphocyten-infiltratie voor en na cytokine therapie onderzocht. Voorlopige data laten een toename van

macrophagen zien na TNF toediening. Humaan TNF bleek niet toxisch te zijn voor de ratten gebruikt in dit proefschrift, hoewel TNF in hoge doseringen is toegediend. Dit fenomeen zou kunnen duiden op de ontwikkeling van neutraliserende antilichamen door de ratten tegen TNF. In een studie met het serum van 12 ratten, uitgevoerd door TNO REP-instituten te Rijswijk, konden deze antilichamen niet worden aangetoond.

In de nabije toekomst zullen door middel van recombinant DNA-technologie nieuwe cytokines gemaakt worden, zoals IFN-alphas (20) en hybride combinatie-moleculen, zoals IFN-gamma en TNF-beta (21). Voor het testen van nieuwe immunotherapieën is de beschikbaarheid van goede preklinische testsystemen zeer belangrijk (22). De diermodellen gebruikt in dit proefschrift lijken hiervoor zeer geschikt. Nieuwe immunotherapeutische modaliteiten, zoals monoclonale antistoffen (23) en nieuwe cytokine combinaties kunnen ook in deze modellen getest worden.

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HLA expression in renal cell carcinoma lesions does not predict the response to interferon therapy. V. Mattijssen, R.J.A. van Moorselaar, P.H. de Mulder, L. Schalkwijk, D.J. Ruiter, submitted

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 15 juli 1961 te Winterswijk. Na het voorbereidend wetenschappelijk onderwijs (V.W.O.) aan het Baudartius College te Zutphen werd in 1979 de studie Geneeskunde aangevangen aan de Katholieke Universiteit te Nijmegen. Van 1 april tot 1 augustus 1986 werkte hij als student-doctor in het Bukumbi Hospital, Bukumbi, Mwanza Region, Tanzania. Het artsexamen werd in juni 1987 afgelegd.

Van juni 1987 tot en met maart 1991 is de auteur als wetenschappelijk medewerker verbonden geweest aan het Urologisch Research Laboratorium (Hoofd: Dr. J.A. Schalken) van de Afdeling Urologie (Hoofd: Prof. Dr. F.M.J. Debruyne) van het Academisch Ziekenhuis Nijmegen. Gedurende deze periode werd het onderzoek verricht dat tot deze dissertatie heeft geleid. Voor het onderzoek gedaan aan niertumoren werd in 1990 de Alken-prijs toegekend.

Sinds april 1991 is hij als arts-assistent algemene heelkunde, in het kader van de opleiding tot uroloog, verbonden aan de Deventer Ziekenhuizen (opleider: dr. P.J. van Elk).

STELLINGEN
behorende bij het proefschrift

CYTOKINE THERAPY IN EXPERIMENTAL UROLOGICAL CANCER

R.J.A. van Moorselaar

Nijmegen, 12 september 1991

I De beste tumormodellen voor het uitvoeren van immunotherapeutische studies bestaan uit spontaan ontstane, syngeen transplanteerbare tumoren.

II Cytokine-therapie is het meest effectief in combinaties en gestart bij een klein tumorvolume.

III De combinatie van tumor necrosis factor en bestraling kan bruikbaar zijn in de behandeling van kanker

IV De antitumor effecten van Adriamycine of VP16 in combinatie met tumor necrosis factor kunnen niet verklaard worden door hun werking op het DNA-topoisomerase-II.

V De antiproliferatieve effecten van cytokines tegen hormoon-afhankelijke en hormoon-onafhankelijke prostaattumoren kunnen leiden tot een nieuwe behandelingsmethode voor deze tumoren

VI De RBT tumorlijnen zijn geschikt voor het onderzoek naar specifieke moleculaire veranderingen geassocieerd met de progressie van blaaskanker.

VII The mouse doctor is a disturbing force to clinicians, because he keeps asking them explanations for their decisions and often such questions cannot be answered rationally.
D W van Bekkum in "Some mouse doctors cure people", IKR-bulletin, april 1991, blz. 3-17

VIII New therapies for advanced renal cell carcinoma should always be based on experimental data rather than just on the availability of new drugs.
R Heicappell and R Ackermann, Urol Res, 18, 357-372, 1990

IX The mouse is an animal which, if killed in sufficient numbers under carefully controlled conditions, will produce a PhD thesis.
Journal of Irreproducible Results

X Zolang de ziekenhuisdirecties werkdagen van meer dan 8 uur accepteren voor arts-assistenten dienen de openingstijden van ziekenhuiscrèches hieraan aangepast te worden.

XI Amateurwielrenners schatten hun intelligentie hoger in dan de professionals gezien de in de eerste groep veel gedragen helm die in de tweede groep zo verguisd is.

XII De kunst is geen reproductie van het zichtbare, zij maakt zichtbaar.
Paul Klee

XIII Gevestigde specialisten zouden zich hun eigen solicitatie-gesprek in herinnering moeten roepen bij het interviewen van aankomende collega's.