Urol Res (1991) 19:91–98

Urological Research

Differential sensitivity of renal cell carcinoma xenografts towards therapy with interferon-alpha, interferon-gamma, tumor necrosis factor and their combinations

A. J.M. C. Beniers, R. J. A. van Moorselaar, W. P. Peelen, F. M. J. Debruyne, and J. A. Schalken

Urological Research Laboratory, Department of Urology, University Hospital Nijmegen, Nijmegen, The Netherlands

Accepted: October 1, 1990

Summary. Whereas cytokine therapy has proven efficacy in the treatment of metastatic renal cell carcinoma (RCC), many questions regarding the use of these drugs remain unanswered. In the present study we evaluated the antiproliferative effects of human recombinant alpha-interferon (IFN), gamma-interferon and tumor necrosis factor-alpha (TNF) on eight human RCC xenografts. In particular, the importance of the administration route, dosage and tumor load was investigated. Response to the cytokines differed widely amongst the different tumors. Of three tested routes of administration (i.v., i.p. and s.c. peritumoral), only the s.c. peritumoral route was effective against tumor growth. After 6 weeks of therapy consisting of 150 or 1,500 units IFN/g given s.c. peritumorally three times a week or 30,000 units TNF/g given five times a week, alpha-IFN treatment resulted in 2%–100% growth inhibition; gamma-IFN, in 7%–80%; and TNF, in 35%–75% as compared with the untreated control. Growth of five of eight tumor lines could be inhibited completely by combinations of IFN and TNF, whereby the tumor dimensions at the beginning of therapy were decisive for the results. In some cases IFNs had optimal doses; however, the antitumor effects of TNF were always dose-dependent. Our studies indicate that the doses at which the optimal direct effects of cytokines are measured are critically dependent on the tumor treated. Although direct effects are only one part of the mode of action of cytokines, our results indicate that dosage of cytokines may need individualisation.

Key words: Renal cell carcinoma – Xenograft – Cytokines – Interferon – Tumor necrosis factor – Nude mouse

Renal cell carcinoma (RCC) is a relatively uncommon malignancy that accounts for 1%–2% of all malignant human tumors [8]. When the tumor is localized, radical nephrectomy can accomplish an effective cure [25, 32]. However, approximately 30% of patients with RCC have clinically manifest metastases at the time of diagnosis [32], and the prognosis for metastatic RCC is poor. The 5-year survival for patients with advanced RCC is $<15\%$ and the mean survival is 1–2 years. Clearly, systemic therapy is necessary in cases of advanced RCC. The efficacy of chemotherapy, however, has proved to be limited. Due to this lack of effective systemic treatments, new methods have been pursued and various forms of hormone therapy [27] or immunological treatment, given alone or in combination with chemo- or radiotherapy [12, 45, 48], have been tested in clinical trials and in animal model systems during the last few decades [11].

The immunological defense mechanism can be stimulated by specific modulations of the immune system that can be categorised into active, passive and adoptive methods [17, 36]. The active method involves stimulation of production of antibodies in patients as well as the cellular immunity; the immune defence can be non-specifically stimulated using, e.g., Bacillus Calmette-Guérin (BCG) [39], *Corynebacterium parvum* [45] or cytokines. The passive method consists of the injection of antibodies into patients. By the adoptive method, immunocytes are specifically stimulated in vitro and reintroduced into patients [1, 5, 14, 26, 46, 56]. Specific and non-specific methods can also be combined and used simultaneously [52].

Interest in biological approaches to the treatment of RCC has been fostered by the identification of unique antigens on the surface of some RCC cells [55] and by observations of spontaneous regression of tumor metastases [19, 31]. The observations suggest that host factors may be capable of modifying the course of RCC [34]. Although the mechanisms of biological response modifiers (BRMs) are not yet well understood, the possibility that cytokines such as interferons (IFNs) and tumor necrosis factor (TNF) might augment a host immune response against RCC has provided a rationale for early clinical trials. Although the clinical responses vary considerably (10%–50%) [9, 21, 23, 33, 35, 41, 47, 54], it is evident that cytokine therapy is effective in approximately 15% of patients with metastasized RCC. The importance of the dosage, administration route and tumor load at the

beginning of therapy have not been extensively evaluated in a preclinical setting. Such insight might lead to optimal treatment regimens.

Transplantation of human RCC into athymic nude mice provides an experimental model [24, 43] in which therapeutic regimens using BRMs may be tested [3, 38, 44]. The T-cell-deficient nude mouse model system, however, does not enable us to study the indirect T-cell-mediated antitumor effects. Nevertheless, the direct effects of BRMs against tumor cells as well as macrophage and NK-cell mobilization can be studied in this in vivo model system [4, 10, 29].

Based on previous in vitro studies [6, 7], the present study tested the effects of recombinant human IFN-alpha, recombinant human IFN-gamma, recombinant human TNF-alpha and their combinations on RCC xenografts growing in nude mice. Different treatment protocols were tested and the role of tumor volume at the start of therapy was evaluated.

Materials and methods

Mice

We used 6-week-old male and female BALB/c nu/nu mice (Bomholt Gård, Ry, Denmark) in these experiments. The animals were adjusted to the experimental environment for 2 weeks prior to the experiments. Groups of five male or female mice were kept in PAG type 2 cages covered with an iso cap (Iffa Credo, France) to ensure sterile conditions. Sterilised sawdust (Iffa Credo Broekman b.v., The Netherlands) was used as bedding material inside the cages. The mice were fed ad libitum with gamma-irradiated (0.9 mR) SRM-A MM food (Hope Farms, Woerden, The Netherlands) and drinking water was acidified with 0.7 ml concentrated HCl/l. A day/night rhythm of 12 h light 12 h darkness and a temperature of 22 °C were maintained. Humidity in the experimental environment was unregulated.

RCC xenografts

Eight different renal tumor xenografts were used in these studies. NC-65 tumor [28] was kindly provided by Dr. J. C. Romijn from the Department of Urology, Erasmus University (Rotterdam, The Netherlands). Seven NU tumors (NU-1, -3, -5, -10, -12, -20 and -22) were established from primary tumors by serial s.c. transplantation (Beniers et al. submitted for publication). The stage of the primary tumors varied between T2 and T4 in the different lines. For the in vivo treatment protocols, we used the 3rd–10th transplant generations of the NU-10, -12, -20 and -22 lines and generations 20–30 of the NC-65 and NU-1, -3 and -5 lines. Tumors were passaged at intervals of 6 weeks for the NU-10, NU-12, NU-20 and NU-22 lines and 4 weeks for the other lines.

Biological response modifiers

Human IFN-alpha, IFN-gamma and TNF-alpha, kindly supplied by Boehringer Ingelheim (Alkmaar, The Netherlands), were produced in *Escherichia coli* by recombinant DNA technology. The specific activity of IFN-alpha and gamma was 3.2×10^8 and 2×10^7 units/mg protein, respectively. It was determined by measurement inhibition of encephalomyocarditis (EMC) virus replication in A549 cells (human lung-myeloma cell line) with reference to the National Institute of Health (NIH) leucocyte IFN-alpha standard Go 23-901-527 and the NIH IFN-gamma standard Gg 23-901-530. The purity of both IFNs was $>98\%$ as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the amount of endotoxin was <1 ng/mg protein for IFN-alpha and ≤ 0.5 ng/mg protein for IFN-gamma as based on the limulus amoebocyte lysate assay. The specific activity of TNF-alpha as determined in the presence of actinomycin D was 6×10^7 units/mg protein (the L-929 cytotoxicity assay). The purity of TNF-alpha was $>99\%$ as determined by SDS-PAGE and it contained ≤ 1 ng endotoxin/mg protein as based on the limulus amoebocyte lysate assay. The drugs were dissolved in the accessory solvent and diluted with unsupplemented RPMI medium (Gibco, Paisley, UK). After dilution, the drugs were stored in 1.2-ml aliquots at −80 °C until used.

In vivo drug testing

Each test group consisted of five mice; male and female animals were used because experiments showed that growth characteristics in control and treatment groups were the same for both sexes (data not shown). Mice were implanted s.c. with 1- to 2-mm cubes of tumor in the right flank. After careful removal of internal necrosis, pieces were cut from s.c. growing tumors that were passaged every 4–6 weeks, depending on the tumor.

Treatment groups were randomized before the start of therapy, and treatment began when tumors had grown to a volume of about 65 mm^3 (10–14 days after implantation) or at 24–48 h after implantation of the tumor pieces. The animals were treated i.p., i.v. (tail vein) or s.c. peritumorally three to five times a week for 6 weeks. Each dose was given in a total volume of 200 µl. IFNs were injected three times a week and TNF, three or five times a week. When animals received both IFNs or IFN + TNF, this combination was given in a single injection. Control mice were treated with unsupplemented RPMI medium three times a week. Treatment protocols and doses for each BRM were determined from the results of preceding experiments, and the starting dose was a combination of 5 ng/g alpha-IFN + 50 ng/g TNF, which produced complete inhibition of colony formation in a soft agar colony-forming assay of the different xenograft lines; the same result was obtained in previous in vitro studies using these tumor lines [6, 7]. In determining this starting dose, we presumed that 1 ml in the in vitro studies equalled 1 g body weight in the animal model system.

Using a sliding caliper, we measured each tumor twice a week in three dimensions: the maximal diameter (L), the diameter at right angles to the length (W) and the thickness (H). The volume of the tumors expressed as the tumor size index (TSI) was calculated by the equation $TSI = L \times W \times H/2$. Each experiment was performed in duplicate.

Statistical analyses

On day 40 of the experiments, TSI values were estimated for each individual mouse by linear regression of the cube roots of all TSI measurements obtained up to that time (Table 1). Since these results showed strong non-normality, the (non-parametric) Kruskal-Wallis test was applied to these values for statistical analysis. Differences were considered to be significant at $P < 0.05$.

Additivity and synergism were defined as follows. A combination treatment was considered to be additive if it produced results that were significantly better than those obtained using each of the single treatments. A combination treatment was considered to be synergistic if its effect exceeded the products of the effects of the single treatments – more precisely, if the product of the median of the combination and control groups was less than the product of the best results (i.e. minimal TSI) obtained in each of the single-treatment groups.

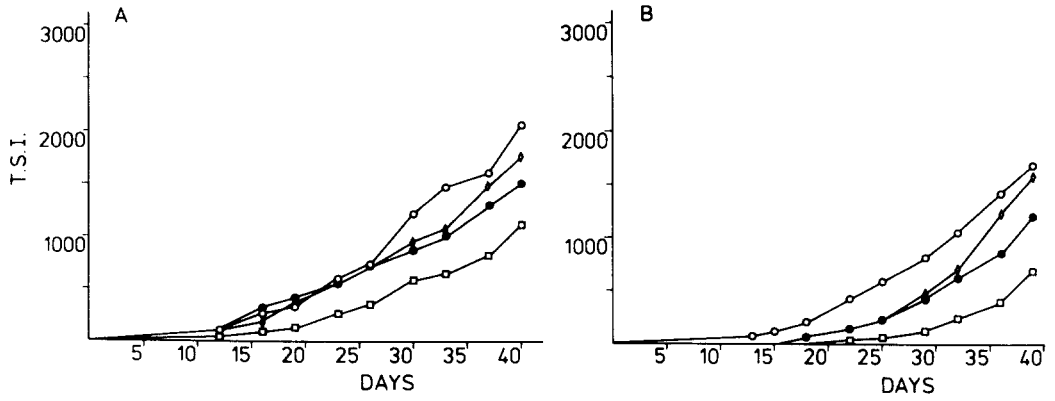

Fig. 1 A, B. Effect of **A** alpha-IFN and **B** a combination of alpha-IFN and TNF on s.c. growth of the NC-65 renal tumor. The BRMs were given s.c. three times a week peritumorally. Treatment started 24 h after s.c. implantation of 1- to 2-mm tumor cubes in the right flank. **A** ○—○, Growth control (treatment 1); □—□, 0.5 ng/g alpha-IFN ($P=0.01$, treatment 2); ●—●, 5 ng/g alpha-IFN ($P=0.30$, treatment 3); ◊—◊, 25 ng/g alpha-IFN ($P=0.21$, treatment 4); Kruskal-Wallis: $P<0.06$. **B** ○—○, growth control (treatment 1); □—□, 0.5 ng/g alpha-IFN, 50 ng/g TNF ($P=0.01$, treatment 2); ●—●, 5 ng/g alpha-IFN, 50 ng/g TNF ($P=0.06$, treatment 3); ◊—◊, 25 ng/g alpha-IFN, 50 ng/g TNF ($P=1.00$, treatment 4); Kruskal-Wallis: $P<0.01$. For the sake of clarity, ranges are not indicated (minimal and maximal values are shown in Table 1)

Results

For purposes of feasibility and efficacy, we used one RCC xenograft to study the effects of alpha-IFN, gamma-IFN and TNF on s.c. tumor growth in a wide range of single and combination tests. The influence of the administration route was also evaluated for this tumor. The NC-65 line was chosen because pilot studies had revealed that it exhibits intermediate sensitivity towards the tested cytokines.

Effect of IFN concentration on tumor growth inhibition

To determine the most effective concentrations of alpha- and gamma-IFN, we performed a series of experiments in which the IFN concentration was varied. We began s.c. peritumoral treatment at 24–48 h after implantation of 1- to 2-mm^3 tumor pieces. NC-65 tumor-bearing animals were treated with three different concentrations of IFN given either alone or in combination with 50 ng/g TNF (i.e. 3,000 units TNF-alpha); the concentrations used were 25, 5 and 0.5 ng/g for alpha-IFN and 400, 80 and 8 ng/g for gamma-IFN (i.e. 7,500, 1,500 and 150 units/g for both IFNs).

Figure 1A, in which the effect of the alpha-IFN concentration on s.c. growth of the NC-65 tumor is depicted, shows that concentrations of 25 and 5 ng/g had no antitumor effect. However, a concentration of 0.5 ng/g exhibited a statistically significant antitumor effect as compared with the growth control ($P=0.01$). A further decrease in the dose resulted in a poorer antitumor effect (data not shown). When TNF was combined with alpha-IFN and the dose of the latter was varied, comparable bell-shaped dose-response curves resulted. Figure 2 illustrates the results of similar experiments performed using gamma-IFN; as with alpha-IFN, bell-shaped dose-response curves were found. Both 80-and 8-ng/g doses resulted in statistically significant inhibition of tumor growth ($P=0.01$), and the lower concentration resulted in the best antitumor effect. Again, when gamma-IFN was given in combination with TNF, the same optimal dose for antitumor efficacy was evident.

Table 1. Median and range of the fitted TSI values calculated on day 40 for the different treatment groups shown in Fig. 1–4 as indicated

Figure	Treatment	Median	Minimum	Maximum
1A	1	2,040	1,660	2,560
	2	1,190	860	1,390
	3	1,590	0	2,450
	4	1,670	0	2,140
1B	1	2,250	1,510	2,650
	2	850	0	1,200
	3	1,410	1,280	2,150
	4	1,950	1,570	2,690
2A	1	2,040	1,660	2,560
	2	750	0	1,200
	3	1,630	0	1,980
	4	1,700	1,530	2,260
2B	1	2,250	1,510	2,650
	2	690	0	850
	3	1,170	950	1,950
	4	1,610	1,380	2,230
3	1	2,040	1,660	2,560
	2	780	580	1,380
	3	760	580	930
	4	190	0	280
	5	510	470	740
4	1	1,810	1,520	2,030
	2	670	610	920
	3	590	380	640
	4	0	0	0
	5	300	200	350

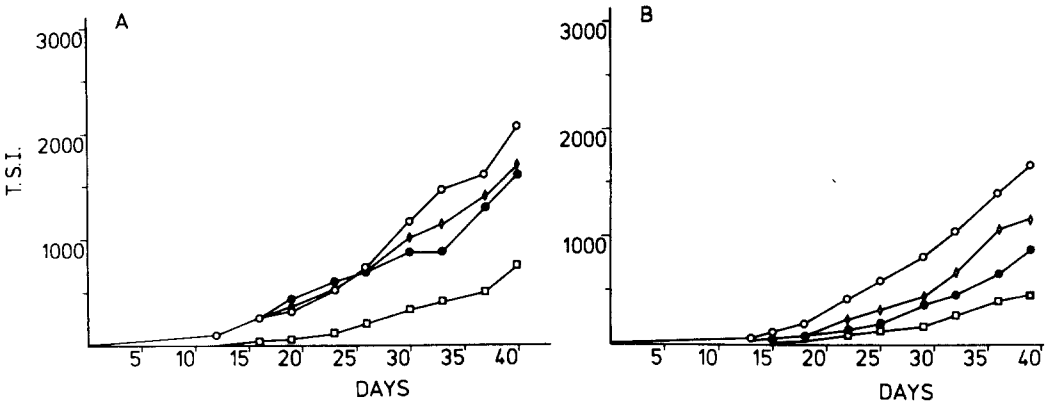

Fig. 2A, B. Effect of A gamma-IFN and B a combination of gamma-IFN and TNF on s.c. growth of the NC-65 renal tumor. The BRMs were given s.c. three times a week peritumorally. Treatment started 24 h after s.c. implantation of 1- to 2-mm tumor cubes in the right flank. A ○—○, Growth control (treatment 1); □—□, 8 ng/g gamma-IFN ($P=0.01$, treatment 2); ●—●, 80 ng/g gamma-IFN ($P=0.04$, treatment 3); ◊—◊, 400 ng/g gamma-IFN ($P=0.21$, treatment 4); Kruskal-Wallis: $P<0.01$. B ○—○, growth control (treatment 1); □—□, 8 ng/g gamma-IFN, 50 ng/g TNF ($P=0.01$, treatment 2); ●—●, 80 ng/g gamma-IFN, 50 ng/g TNF ($P=0.02$, treatment 3); ◊—◊, 400 ng/g gamma-IFN, 50 ng/g TNF ($P=0.09$, treatment 4); Kruskal-Wallis: $P<0.01$. For the sake of clarity, ranges are not indicated (minimal and maximal values are shown in Table 1)

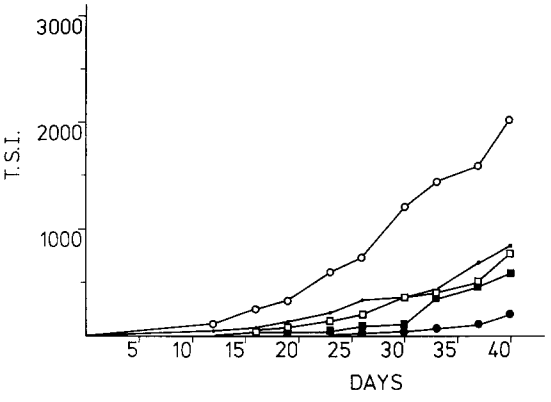

Fig. 3. Effect of TNF concentration in combination with alpha- or gamma-IFN on s.c. growth of the NC-65 renal tumor. The BRMs were given s.c. three times a week peritumorally. Treatment started 24 h after s.c. implantation of 1- to 2-mm tumor cubes in the right flank. ○—○, Growth control (treatment 1); •—•, 0.5 ng/g alpha-IFN, 50 ng/g TNF ($P=0.01$, treatment 2); □—□, 8 ng/g gamma-IFN, 50 ng/g TNF ($P=0.01$, treatment 3); ●—●, 0.5 ng/g alpha-IFN, 500 ng/g TNF ($P=0.01$, treatment 4); ■—■, 8 ng/g gamma-IFN, 500 ng/g TNF ($P=0.01$, treatment 5); Kruskal-Wallis: $P<0.001$). For the sake of clarity, ranges are not indicated (minimal and maximal values are shown in Table 1)

Effect of TNF concentration and dosage on tumor growth inhibition

To determine the TNF concentration and dosage resulting in the best antiproliferative effect, we varied these two parameters. Figure 3 shows the results of an experiment in which the antitumor effects of TNF (50 and 500 ng/g) given in combination with optimal alpha- and gamma-IFN doses of 0.5 and 8 ng/g, respectively, on the NC-65 tumor were examined. Both of the IFNs as well as TNF were given s.c. three times a week peritumorally. Unlike the typical bell-shaped dose-response curves obtained for the IFNs, the antitumor effect of TNF was dose-dependent (Fig. 3). TNF concentrations of 250 ng/g resulted in intermediate tumor growth inhibition, and doses of 25 ng/g produced lower tumor growth inhibition than did a concentration of 50 ng/g (data not shown).

Because no complete tumor growth inhibition could be achieved with IFN/TNF combinations when both drugs were given three times a week, we varied the TNF dosage. Figure 4 shows an example of an experiment using the NC-65 tumor in which the IFNs were injected three times a week and TNF was given five times a week. Therapy was started at 24–48 h after s.c. tumor implantation and the first treatment always consisted of both BRMs given in a single injection. Administration of TNF five times a week resulted in antitumor effects that were significantly better than those observed following three doses of this BRM a week (Fig. 4). The combination of 0.5 ng/g alpha-IFN given three times a week with 500 ng/g TNF injected five times a week resulted in complete inhibition of tumor development. The mice were kept alive for 8 months after treatment, and no tumor growth could be detected by that time.

Effect of the route of administration on growth inhibition by alpha-IFN, gamma-IFN, TNF and their combinations

Experiments using the NC-65 tumor in which the IFNs and TNF were injected i.p. or i.v. showed no significant antitumor effects on the s.c. growing tumor; the latter route of administration even resulted in a slight increase in tumor growth.

Effects of alpha-IFN, gamma-IFN, TNF and their combinations on s.c. tumor growth of eight different RCC xenografts

From the results obtained using the NC-65 tumor, we chose ten treatment regimens; five single-agent therapies and five combination regimens so as to evaluate their

Table 2. Effects of alpha-IFN, gamma-IFN, TNF and their combinations on s.c. tumor growth of eight different RCC xenografts

Treatment	NC-65 growth (%)	NU-1 growth (%)	NU-3 growth (%)	NU-5 growth (%)	NU-10 growth (%)	NU-12 growth (%)	NU-20 growth (%)	NU-22 growth (%)
Control	100	100	100	100	100	100	100	100
alpha-IFN 0.5	74	98	64	98	63	28	76	31
alpha-IFN 5	96	51	75	39	63	0	69	17
gamma-IFN 8	61	61	58	93	37	34	79	63
gamma-IFN 80	80	62	57	85	46	21	81	70
TNF 500	60	38	56	28	30	50	65	23
alpha-IFN 0.5/gamma-IFN 8	45	46	34	67	21	10	61	21
alpha-IFN 0.5/TNF 500	0	30	24	7	0	5	28	5
alpha-IFN 5/TNF 500	12	5	31	0	0	0	16	0
gamma-IFN 8/TNF 500	19	19	11	13	0	13	22	11
gamma-IFN 80/TNF 500	21	21	24	9	0	0	31	13

The IFNs were given three times a week and TNF, five times a week. Treatment started 24 h after implantation of 1- to 2-mm tumor cubes and the BRMs were injected s.c. peritumorally. BRM concentrations are given in ng/g. Combinations were given in one single injection. *P* values for the different tumors were 0.01 for all combination treatments except the alpha/gamma-IFN combination in the NU-5 tumor ($P = 0.14$). Kruskal-Wallis: $P < 0.0001$ for each xenograft

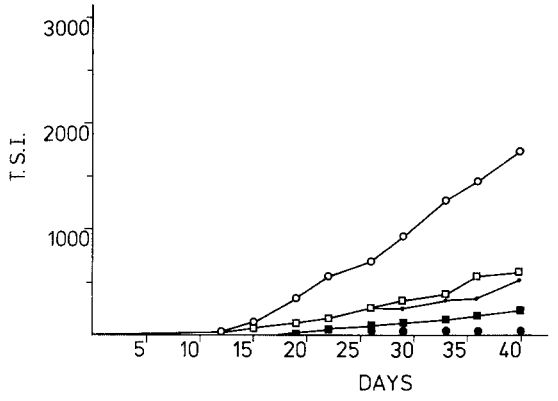

Fig. 4. Effect of the dosage of TNF, given in combination with fixed alpha- and gamma-IFN doses on s.c. growth of the NC-65 renal tumor. The IFNs were given three times a week and TNF, three or five times a week. Treatment started 24 h after implantation of 1- to 2-mm cubes and the BRMs were injected s.c. peritumorally. ○—○, Growth control (treatment 1); □—□, 0.5 ng/g alpha-IFN, 500 ng/g TNF (3×/week, treatment 2; $P = 0.01$); •—•, 8 ng/g gamma-IFN, 500 ng/g TNF (3×/week, treatment 3; $P = 0.01$); ●—●, 0.5 ng/g alpha-IFN, 500 ng/g TNF (5×/week, treatment 4; $P = 0.01$); ■—■, 8 ng/g gamma IFN, 500 ng/g TNF (5×/week, treatment 5; $P = 0.01$); Kruskal-Wallis: $P < 0.0001$. For the sake of clarity, ranges are not indicated (minimal and maximal values are given in Table 1)

effects on a set of eight human RCC xenografts. All experiments were performed as described in the previous sections. The results a summarized in Table 2. The IFNs were given three times a week and TNF, five times a week, beginning at 24–48 h after tumor implantation.

The growth of the NU-1 line, a fast-growing tumor (tumor-doubling time 4 days), could not be completely inhibited by any of the combinations tested. The combination of 5 ng/g alpha-IFN + 500 ng/g TNF resulted in the strongest inhibition of tumor growth with 5% growth being observed in this groups as compared with controls. In contrast to experiments using the NC-65 tumor, in the NU-line the higher concentration of alpha-IFN was more active than the lower dose following both single-agent and combination administration. However, following treatment with gamma-IFN, no statistically significant differences in antitumor effects were observed between the 8- and 80-ng/g treatment groups, whether this agent was given alone or in combination with TNF.

The NU-3 line appeared to be one of the least sensitive tumors, with the maximal antitumor effects of the combination of 8 ng/g gamma-IFN + 500 ng/g TNF being 11% growth as compared with that in controls. This tumor was most sensitive to gamma-IFN and, although no difference in antitumor activity was observed between the 8- and 80 ng/g treatment groups, the combination with TNF resulted in differences in antitumor effects between to two gamma-IFN doses, with the low concentration producing the highest antitumor activity.

Development of the NU-5 tumor was completely inhibited by a combination of 5 ng/g alpha-IFN given three times a week and 500 ng/g TNF injected five times a week. The percentage of tumor growth was lower after treatment with the higher doses of both alpha- and gamma-IFN, whether given alone or in combination with TNF, suggesting a different optimal IFN dose for this tumor.

The NU-10 tumor was very sensitive to the IFN-TNF combinations, all of which resulted in complete inhibition of tumor development. No difference in antitumor effect was found between the 0.5- and 5-ng/g alpha-IFN test groups, although this tumor appeared to be more sensitive to the gamma-IFN dose of 8 ng/g than to the 80-ng/g concentration.

An alpha-IFN dose as low as 5 nglg resulted in complete inhibition of NU-12 tumor development. This tumor appeared to be moderately sensitive to 500 ng/g TNF monotherapy, but combinations of 5 ng/g alpha-IFN (as expected due to the effect of monotherapy at this dose) and 80 ng/g gamma-IFN with TNF both resulted in complete inhibition of tumor development. As with the NU-5 tumor, the highest of both alpha- and gamma-IFN

combinations testes produced the best results with regard to NU-12 tumor growth inhibition.

Like the NU-3 line, the NU-20 tumor was rather insensitive to the cytokines. The optimal dose of alpha-IFN should be >0.5 ng/g because the higher dose, whether given alone or in combination with TNF, resulted in reduced tumor growth as compared with the low dose tested. Also, this tumor was more sensitive to the lower gamma-IFN dose tested. Although no difference in sensitivity was observed between the doses tested in monotherapy experiments, the differences noted following combination therapy with TNF were quite pronounced.

Development of the NU-22 tumor was also more strongly inhibited by s.c. peritumoral treatment with 5 ng/g alpha-IFN than with a dose of 0.5 ng/g. Treatment of this tumor with the latter dose resulted in 31% growth as compared with that observed in controls whereas treatment with 5 ng/g alpha-IFN resulted in only 17% growth; thus, the optimal alpha-IFN dose does not lie within the very low dose range. The combination of 5 ng/g alpha-IFN and 500 ng/g TNF produced complete inhibition of tumor development. Gamma-IFN was less active against this tumor, and no significant difference in antiproliferative action was noted between the two doses, whether tested alone or in combination with TNF.

Effect of tumor volume on growth inhibition by combinations of alpha-IFN, gamma-IFN and TNF

To establish the effects of tumor volume on the efficacy of the treatment, s.c. growing tumors showing TSI values of between 50 and 500 were treated with their optimal IFN/TNF combination doses. No growth-inhibitory effects could be measured after treatment had begun in tumors exhibiting a TSI of 500, and only reduced growth-inhibitory effects were noted after treatment had been started in tumors showing TSI values of between 50 and 100; the latter involved the NC-65, NU-1, NU-3, NU-10 and NU-12 tumors. Treatment of tumors with their optimal combination doses (as indicated in Table 2) resulted in a 5- to 10-fold reduction in growth inhibition when the TSI values for the tumors was 50–70. Treatment of tumors showing TSI values of 100 further reduced the growth-inhibitory effects of the BRM combinations. Moreover, in the NC-65 tumor, no antitumor effect was evident when BRM therapy was started at TSI values of 100 (data not shown).

Discussion

Patients with renal cell carcinoma (RCC) usually present clinically at a late stage of disease progression, i.e. 30% have proven metastases at initial diagnosis. This proportion increases further when one takes into account that follow-up examinations of patients who have undergone nephrectomy for clinically localized disease reveal a number of subjects who apparently had micrometastases at initial diagnosis. It is therefore evident that systemic therapeutic regimens are necessary for the treatment of RCC patients. Whereas renal tumors are refractory to nearly all forms of chemotherapy, immunotherapy has proved to be promising in the treatment of metastatic RCC.

The use of cytokines such as IFNs and TNF was evaluated in this study. Although clinical trials have established that these BRMs can be effective in the treatment of metastatic RCC, many questions remain unanswered, one of which involves optimal dose, dosage and route of administration for IFN and TNF. The answers to this and to the question as to whether these parameters have to be optimised for individual patients clearly necessitate the use of animal model systems. We used human RCC tumors transplanted into nude mice, in which only the direct and the macrophage- and NK-cell-mediated effects of these cytokines can be evaluated.

The results presented in this paper show that the tumors exhibit different sensitivities to the various BRMs. Depending on the tumor, s.c. development of five tumors could be completely inhibited by different combinations of both of the IFNs with TNF. The s.c. development of only one tumor, the NU-12 lines could be completely inhibited by alpha-IFN monotherapy. However, s.c. growth of three of the tumors tested could not be inhibited completely.

The highest concentration of alpha-IFN was usually the most effective. In five tumors (NU-1, -5, -12, -20 and -22), growth was inhibited more strongly by 5 ng/g alpha-IFN, whereas in two others (NC-65 and NU-3) the lower concentration was the most effective. In the NU-10 tumor, no difference in sensitivity was evident between the doses tested. In contrast, gamma-IFN was usually more effective at the lower concentration tested (8 ng/g). The growth of three tumors (NC-65, NU-10, NU-22) was more strongly inhibited at the lower dose, whereas no significant difference was found for three others (NU-1, NU-3 and NU-20). However, the NU-3 and NU-20 tumors proved to be more sensitive to the lower gamma-IFN dose when it was given in combination with TNF. In two tumors (NU-5 and NU-12), the highest gamma-IFN dose was the most effective, whether given alone or in combination with TNF.

The data resulted in bell-shaped dose-response curves for gamma-IFN in the dose range tested, and similar effects were seen for alpha-IFN in a minority of cases. The antiproliferative effect of these drugs is clearly different, in the eight xenograft lines tested the optimal doses determined under our experimental conditions varied 10-fold. This indicates an optimal IFN dose for each individual tumor, which implies that parameters must be evaluated that can be used to optimise treatment regimens. Serum $beta_2$-microglobulin and neopterin could be such candidates [2].

The finding that lower doses of alpha- and gamma-IFN can be more effective could be advantageous for clinical use, since high concentrations of IFNs can easily lead to immunosuppression [16, 42, 53]. Combinations of the lowest tested doses of both IFNs showed additive effects on the NC-65 and NU-5, -10, -12 and -22 tumors. Synergistic effects were never found following treatment with a combination of both IFNs. The antitumor effects of

combinations of alpha- or gamma-IFN with TNF, however, usually proved to be either additive or synergistic (data not shown).

The direct antiproliferative effect of TNF is dose-dependent. The treatment schedule also affects the antiproliferative effects of TNF, as a protocol involving treatment five times a week results in further tumor growth inhibition as compared with a protocol by which TNF is given three times a week. As these effects were noted following combination treatment with alpha- or gamma-IFN as well as after TNF monotherapy, they are not directly related to a combination with IFN.

The dose-response curve for TNF-alpha is similar to that of most chemotherapeutic agents, i.e. the higher the dose, the better the antitumor effect. The toxicity of TNF is a major problem in clinical situations; thus, only low doses should be given to patients [13, 15, 49]. Taking into account the different area: volume ratios [20], when the dose used to treat nude mice is translated to the human situation, it appears that the tolerance of nude mice for human recTNF is at lest 10-fold that of humans to human recTNF. The treated mice did not show any signes of toxicity when human recTNF was injected s.c. Whether the minor differences between human and murine TNF molecules [18, 40, 50] can explain the occurrence of such a dramatic reduction in toxicity without a corresponding reduction in the direct antitumor effects remains speculative; however, the implications thereof would be profound.

Because combinations of TNF with IFNs can have a synergistic antiproliferative effect, it is of greatest importance that the effect of TNF is enhanced by its combination with either alpha- or gamma-IFN. Thus, complete growth inhibition could be provoked in most xenografts tested in the present study; moreover, the effect following combination therapy was different in the eight xenograft lines tested.

The effect of initial tumor volume was evaluated in five lines. It appeared that at higher tumor volumes, i.e. at a TSI of > 50, only partial growth-inhibitory effects of the BRMs were evident. The clinical implication would be that cytokine treatment is applicable only to the group of patients with clinically localized disease who have micrometastases. Patients at relatively high risk (T3) could be candidates for such adjuvant treatment regimens. However, for removal of the primary tumor itself, radical nephrectomy remains to treatment of choice [32].

Previous clinical trials have revealed that cytokine treatment can provoke tumor regression [30, 51], indicating that indirect effects (i.e. immune system-mediate) can play a crucial role in the antitumor activity of such treatment. Inclusion of these effects in animal model systems now seems possible by the use of SCID mice, in which the human immune system can be partly reconstituted [22, 37]. Thus, more insight could be gained into the complete spectrum of cytokine-mediated antitumor effects and the usefulness of these agents could be studied to a greater extent.

Acknowledgements. The authors would like to thank Mr. J. Koedam and Mrs. M. Derks for their excellent performance of animal treatment. We are grateful to Dr. G. Borm and Dr. A. Theeuwes for their statistical analyses of the results. This work was supported by grants from the Netherlands Kidney Foundation (C85-563 and C89-838), Boehringer Ingelheim (FRG), the Maurits and Anna de Kock Foundation and the Nijbakker-Morra Foundation.

References

1. Alexander J, Rayman P, Edinger M, Connelly R, Tubbs R, Bukowski R, Pontes E, Finke J (1990) TIL from renal-cell carcinoma: restimulation with tumor influences proliferation and cytolytic activity. Int J Cancer 45:119
2. Aulitzky W, Gastl G, Aulitzky WE, Herold M, Kemmler J, Mull B, Frick J, Huber C (1990) Successful treatment of metastatic renal cell carcinoma with a biologically active dose of recombinant interferon-gamma. J Clin Oncol 7:1875
3. Balkwill FR, Proietti E (1986) Effects of mouse interferon on human tumour xenografts in the nude mouse host. Int J Cancer 38:375
4. Bancroft GJ, Sheehan KCF, Schreiber RD, Unanue ER (1989) Tumor necrosis factor is involved in the T cell-independent pathway of macrophage activation in SCID mice. J Immunol 143:127
5. Belldegrun A, Muul LM, Rosenberg SA (1988) Interleukin 2 expanded tumor-infiltrating lymphocytes in human renal cell cancer: isolation, characterization, and antitumor activity. Cancer Res 48:206
6. Beniers AJMC, Peelen WP, Hendriks BT, Schalken JA, Romijn JC, Debruyne FMJ (1988) In vitro antiproliferative efficacy of interferon-alpha, -gamma and tumor necrosis factor on two human renal tumor xenografts. Urol Res 16:309
7. Beniers AJMC, Moorselaar RJA van, Peelen WP, Hendriks BT, Otto U, Schalken JA, Debruyne FMJ (1989) In vitro sensitivity of three human renal tumor xenografts towards tumor necrosis factor and alpha- and gamma-interferon. In: Rübben H, Jocham D, Jacobi GH (eds) Investigative urology, vol 3. Springer, Berlin Heidelberg New York, p 30
8. Bissada N (1977) Renal cell carcinoma. Surg Gynecol Obstet 145:97
9. Bonnem EM, Spiegel RJ (1984) Interferon-alpha: current status and future promise. J Biol Response Mod 3:580
10. Brunda MJ, Bellantoni D, Sulich V (1987) In vivo anti-tumor activity of combinations of interferon alpha and interleukin-2 in a murine model. Correlation of efficacy with the induction of cytotoxic cells resembling natural killer cells. Int J Cancer 40:365
11. Burgers JK, Marshall FF, Isaacs JT (1989) Enhanced anti-tumor effects of recombinant human tumor necrosis factor plus VP-16 on metastatic renal cell carcinoma in a xenograft model. J Urol 142:160
12. Creagan ET, Buckner JC, Hahn RG, Richardson RR, Schaid DJ, Kovach JS (1988) An evaluation of recombinant leukocyte A interferon with aspirin in patients with metastatic renal cell cancer. Cancer 61:1787
13. Creagan ET, Kovach JS, Moertel CG, Frytak S, Kvols LK (1988) A phase I clinical trial of recombinant human tumor necrosis factor. Cancer 62:2467
14. Economu JS, Colquhoun SD, Anderson TM, McBride WW, Golub S, Holmes EC, Morton DL (1988) Interleukin-1 and tumor necrosis factor production by tumor-associated mononuclear leukocytes and peripheral mononuclear leukocytes in cancer patients. Int J Cancer 42:712
15. Feinberg B, Kurzrock R, Talpaz M, Blick M, Saks S, Gutterman JU (1988) A phase I trial of intravenously-administered recombinant tumor necrosis factor-alpha in cancer patients. J Clin Oncol 6:1328
16. Figlin RA, deKernion JB, Mukamel E, Palleroni AV, Itri LM, Sarna GP (1988) Recombinant interferon alpha-2a in metastatic renal cell carcinoma: assessment of antitumor activity and anti-interferon antibody formation. J Clin Oncol 6:1604

17. Foon KA (1989) Biological response modifiers: the new immunotherapy. Cancer Res 49:1621
18. Fransen L, Ruysschaert M-R, Heyden JV van der, Fiers W (1986) Recombinant tumor necrosis factor: species specificity for a variety of human and murine transformed cell lines. Cell Immunol 100:260
19. Freed SZ, Halperin JP, Gordon M (1977) Idiopathic regression of metastases from renal cell carcinoma. J Urol 118:538
20. Freireich EJ, Gehan EA, Rall DP, Schmidt LH, Skipper HE (1966) Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. Cancer Chemother Rep 50:219
21. Fujita T, Asano H, Naide Y, Ono Y, Ohshima S, Suzuki K, Aso Y, Ariyoshi Y, Fikushima M, Ota K (1988) Antitumor effects of human lymphoblastoid interferon on advanced renal cell carcinoma. J Urol 139:256
22. Fulop GM, Phillips RA (1989) Use of SCID mice to identify and quantitate lymphoid-restricted stem cells in long-term bone marrow cultures. Blood 74:1537
23. Garnick MB, Reich SD, Maxwell B, Coval-Goldsmith S, Richie JP, Rudnick SA (1988) Phase I/II study of recombinant interferon gamma in advanced renal cell carcinoma. J Urol 139:251
24. Giovanella BC, Stehlin JS, Shepard RC, Williams LJ (1983) Correlation between response to chemotherapy of human tumors in patients and in nude mice. Cancer 52:1146
25. Golimbu M, Al-Askari S, Tessler A, Morales P (1986) Aggressive treatment of metastatic renal cancer. J Urol 136:805
26. Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA (1982) The lymphokine activated killer cell phenomenon: lysis of natural killer resistant fresh solid tumor cells by interleukin 2 activated autologous human peripheral blood lymphocytes. J Exp Med 155:1823
27. Harris DT (1983) Hormonal therapy and chemotherapy of renal-cell carcinoma. Semin Oncol 10:422
28. Hoehn W, Schroeder FH (1978) Renal cell carcinoma: two new cell lines and a serially transplantable nude mouse tumor (NC-65). Invest Urol 16:106
29. Hoskin DW, Stankova J, Anderson SK, Roder JC (1989) A functional and phenotypic comparison of murine natural killer (NK) cells and lymphokine-activated killer (LAK) cells. Int J Cancer 43:940
30. Jaffe HS, Herberman RB (1988) Rationale for recombinant human interferon-gamma adjuvant immunotherapy for cancer. J Natl Cancer Inst 80:616
31. deKernion JB, Berry D (1980) The diagnosis and treatment of renal cell carcinoma. Cancer 45:1947
32. deKernion JB, Mukamel E (1987) Selection of initial therapy for renal cell carcinoma. Cancer 60:539
33. Koiso K (1987) Phase II study of recombinant human interferon gamma (S-6810) on renal cell carcinoma. Cancer 60:929
34. Krown SE (1985) Therapeutic options in renal-cell carcinoma. Semin Oncol 12 [suppl 5]:13
35. Krown SE (1987) Interferon treatment of renal cell carcinoma. Cancer 59:647
36. Mathe G, Amiel J-L, Schwarzenberg L, Schneider M (1970) The immunological approach to cancer treatment. J R Coll Physicians Lond 5:62
37. McCune JM, Namikawa R, Kaneshima H, Shultz LD, Lieberman M, Weissman IL (1988) The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. Science 241:1632
38. Mcintosh JK, Mulé JJ, Krosnick JA, Rosenberg SA (1989) Combination cytokine immunotherapy with tumor necrosis factor alpha, interleukin 2, and alpha-interferon and its synergistic antitumor effects in mice. Cancer Res 49:1408
39. Morales A, Eidinger D (1976) Bacillus Calmette-Guérin in the treatment of adenocarcinoma of the kidney. J Urol 115:377
40. Mukavitz Kramer S, Aggarwal BB, Tessalu TE, McCabe SM, Ferraiolo BL, Figari IS, Palladino MA (1988) Characterization of the in vitro and in vivo species preference of human and murine tumor necrosis factor-alpha. Cancer Res 48:920
41. Muss HB, Constanzi JJ, Leavitt R, Williams RD, Kempf RA, Pollard R, Ozer H, Zekan PJ, Grunberg SM, Mitchell MS, Caponera M, Gavigan M, Ernest ML, Venturi C, Greiner J, Spiegel RJ (1987) Recombinant alfa interferon in renal cell carcinoma: a randomized trial of two routes of administration. J Clin Oncol 5:286
42. Öberg K, Alm G, Magnusson A, Lundqvist G, Theodorsson E, Wide L, Wilander E (1989) Treatment of malignant carcinoid tumors with recombinant interferon alpha-2b: development of neutralizing interferon antibodies and possible loss of antitumor activity. J Natl Cancer Inst 81:531
43. Otto U, Klöpel G, Baisch H (1984) Transplantation of human renal cell carcinoma into NMRI nu/nu mice: I. Reliability of an experimental tumor model. J Urol 131:130
44. Ramani P, Balkwill FR (1988) Human interferons inhibit experimental metastases of a human melanoma cell line in nude mice. Br J Cancer 58:350
45. Regenass U, Müller M, Curschellas E, Matter A (1987) Anti-tumor effects of tumor necrosis factor in combination with chemotherapeutic agents. Int J Cancer 39:266
46. Rosenberg SA (1985) Lymphokine activated killer cells; a new approach to the immunotherapy of cancer. J Natl Cancer Inst 75:595
47. Sarna G, Figlin R, Dekernion JB (1987) Interferon in renal cell carcinoma. Cancer 59:610
48. Sersa G, Willingham V, Milas L (1988) Anti-tumor effects of tumor necrosis factor alone or combined with radiotherapy. Int J Cancer 42:129
49. Sherman ML, Spriggs DR, Arthur KA, Imamura K, Frei E, Kufe DW (1988) Recombinant human tumor necrosis factor administered as a five-day continuous infusion in cancer patients: phase I toxicity and effects on lipid metabolism. J Clin Oncol 6:344
50. Smith RA, Kirstein M, Fiers W, Baglioni C (1986) Species specificity of human and murine tumor necrosis factor. J Biol Chem 262:14871
51. Swanson DA, Quesada JR (1988) Interferon therapy for metastatic renal cell carcinoma. Semin Surg Oncol 4:174
52. Tallberg T, Tykkä H (1986) Specific active immunotherapy in advanced renal cell carcinoma: a clinical long-term follow-up study. World J Urol 3:234
53. Teichmann JV, Sieber G, Ludwig W-D, Ruehl H (1989) Immunosuppressive effects of recombinant interferon-alpha during long-term treatment of cancer patients. Cancer 63:1990
54. Trump DL, Ravdin PM, Borden EC, Magers CF, Whisnant JK (1990) Interferon-alpha-n1 and continuous infusion vinblastine for treatment of advanced renal cell carcinoma. J Biol Response Mod 9:108
55. Ueda R, Ogata S-I, Morrissay DM (1981) Cell surface antigens of human renal cancer defined by mouse monoclonal antibodies: identification of tissue-specific kidney glycoproteins. Proc Natl Acad Sci USA 78:5122
56. Wang JCL, Walle A, Novogrodsky A, Suthanthiran M, Silver RT, Bander NH, Rubin AL, Stenzel KH (1989) A phase II clinical trial of adoptive immunotherapy for advanced renal cell carcinoma using mitogen-activated autologous leukocytes and continuous infusion interleukin-2. J Clin Oncol 7:1885

A. J. M. C. Beniers, PhD
Urological Research Laboratory
Department of Urology
University Hospital Nijmegen
Geert Grooteplein Zuid 16
P.O. Box 9101
6500 HB Nijmegen
The Netherlands