Clinical and biological effects of Interleukin 12 in patients with renal cell carcinoma

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Clinical and Biological Effects of Interleukin 12 in Patients with Renal Cell Carcinoma

Klinische en biologische effecten van IL-12 bij patiënten met niercelkanker

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Voor Els en Adine

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Chapter 1

Introduction

Immunotherapy of cancer

The immunotherapy of cancer is based on the assumption that the immune system can be stimulated to recognize cancer and eradicate tumor. The course of most human cancers indicates that natural protective immunity against cancer either is non existent or fails. Theoretical models of immune activation propose that the immune system has evolved to recognize self from non-self [Langman et al, 2000], and that immune activation is initiated when the immune system senses or recognizes danger [Matzinger, 2001]. According to these models, the immune tolerance that may exists for cancer cells has been hypothesized to develop due to predominant expression of self antigens on tumors or lack of recognition of non-self on tumors. Alternatively, tolerance may be due to lack of "danger" signals during the early development of cancer. The goal of cancer immunotherapy is to exploit the immune system in the battle against cancer.

One of the approaches of immunotherapy consists of systemic administration of cytokines. Cytokines are soluble proteins involved in cellular communication, initially identified as products of cells of the immune system. Large scale production of cytokines by recombinant DNA technology has enabled the study of their anti-tumor efficacy. Interleukin (IL-)12 is a cytokine with important immunoregulatory functions and strong anti-tumor effects in animal models.

Renal cell cancer is one of the few malignancies that has shown susceptibility to cytokine immunotherapy. While conventional chemotherapy has a low efficacy, with response rates of 15% at the best [reviewed by Ruiz et al, 2000], therapy with interferon (IFN) α, or the combination of IL-2 and IFN-α, with or without 5-fluorouracil, has resulted in response rates between 15 and 36%. [reviewed by Motzer et al, 2000; Atzpodien et al, 2000]. Still, effective treatment is not available for the majority of patients with metastatic disease, and therefore, new treatment strategies must be developed.

IL-12: structure, production and receptors

IL-12 was discovered 1989, and initially called "cytotoxic lymphocyte maturation factor" [Stern et al, 1990] or "natural killer (NK) cell stimulating factor" [Kobayashi et al, 1989]. IL-12 is composed of two disulfide-linked sub-units with molecular weights of 35kDa (p35) and 40kDa (p40), respectively [Podlaski et al, 1992]. P35 and p40 are structurally unrelated and their genes have been mapped to separate chromosomes, i.e. 3p12-3q13.2 and 5q31-q33

[Sieburth et al, 1992]. The p40 sequence is not homologous to any other known cytokine and has sequence homology with the extra-cellular domain of the IL-6 receptor [Gearing et al, 1991], whereas the p35 sub-unit has structural similarities with IL-6 itself and granulocyte-colony- stimulating factor (G-CSF) [Merberg et al, 1992]. Therefore it has been proposed that IL-12 resembles a secreted, disulfide-linked complex of a cytokine with its own receptor. Various cells produce IL-12, such as phagocytic cells, dendritic cells and B lymphocytes [reviewed by Trinchieri 1996; Heufler et al, 1996; Schultze et al, 1999].

The production of IL-12 is strongly stimulated by infectious pathogens and their products [D'Andrea et al, 1992; Sato et al, 1996]. Additionally, CD40 ligand-receptor interactions, between T lymphocytes and antigen presenting cells, result in the production of IL-12 by the latter [Shu et al, 1995].

High affinity receptors for IL-12 are composed of two sub-units, designated β1 and β2 [Presky et al, 1996]. In humans, each subunit binds IL-12 with only low affinity, while co-expression is required for the generation of high affinity binding. The β2 subunit is more restricted in its distribution [Presky et al, 1996]. Dual expression of the sub-units has been shown on NK- and T cells. Several other cells, such as neutrophils, dendritic cells, B lymphocytes and eosinophils, express the β1 sub-unit and were shown to respond to IL-12 in-vitro [Desai et al, 1992; Nutku et al, 2001; Nagayama et al, 2000; Airoldi et al, 2000]. Signal transduction through the high affinity receptors on lymphocytes involves tyrosine phosphorylation of the Tyk2 and Jak2 kinases and of the transcription factors STAT3 and STAT4 [Bacon et al, 1995a and 1995b; Jacobson et al, 1995].

Biological activities in vitro

IL-12 has several biological properties that may have anti-tumor effects, but the most important one is probably enhancement of the cellular immune reactivity by directing T helper cell differentiation in favor of T helper type 1 (Th1) responses [Germann et al, 1993; Trinchieri, 1996; Heufler et al, 1996]. Th1 cells produce IFN-γ and IL-2 and promote the cell mediated immune response, that is considered the most important effector mechanism in the eradication of tumors. IL-12 promotes the commitment of naive T cells, during their encounter with antigen, into Th1 cells, and in addition, stimulates maximum secretion of IFN-γ by Th1 cells and promotes the activation of memory Th1 cells [Manetti et al, 1993; Germann et al, 1993; Heufler et al, 1996]. Another important property of IL-12 is the ability to strongly stimulate activated T and NK

cells to produce IFN-γ. In fact, IFN-γ is considered the most important mediator of the biological effects of IL-12 administration in vivo [Wu et al, 1993; Brunda et al, 1995a; Nastala et al, 1994]. In addition, IL-12 induces other cytokines, such as tumor necrosis factor-α (TNF-α), granulocyte-macrophage-colony-stimulating factor (GM-CSF), IL-2, IL-3, IL-8 and IL-10, that can regulate activation and migration of various immune cells [Trinchieri, 1996]. In vitro, IL-12 also stimulates NK- and lymphokine activated killer cell non-specific lytic activity against tumor cells in culture [Kobayashi et al, 1989] and promotes activation of specific cytolytic T cells [Gately et al, 1992].

Animal studies

IL-12 administration had potent anti-tumor effects in several murine tumor models, including murine renal cell cancer [reviewed by Brunda et al, 1996]. IL-12 was administered either systemically by intra-peritoneal injection or locally, by fibroblasts genetically engineered to produce IL-12. IL-12 administration resulted in complete tumor regression of established tumors and their metastases, reduced spontaneous metastases and induced resistance to re-challenge with the same tumor after initial cure. In distinct tumor models, the mechanisms responsible for the anti-tumor effect were different, i.e. depended on the model studied. Cellular depletion studies and studies in knockout mice have demonstrated that NK cells , CD8+ and CD4+ T cells, or a combination of these are most frequently involved in the anti-tumor effect in animal models [Brunda et al, 1996]. In addition, it was shown, that although IFN-γ was required for optimal anti-tumor responses in most models, IFN-γ in itself was not sufficient to mediate the anti-tumor effects [Brunda et al, 1995a]. IL-12 was also shown to inhibit tumor associated angiogenesis [Voest et al, 1995; Sgadari et al, 1996]. Pre-clinical studies with human IL-12 were performed in severe combined immunodeficiency (SCID) mice. Transfer of human effector cells, such as NK and cytotoxic T cells, in combination with treatment with human IL-12 resulted in prolonged survival of tumor bearing mice [Cesano et al, 1994, 1995]. The anti-tumor efficacy of IL-12 has been compared with other cytokines with established efficacy in cancer. IL-12 had greater activity than IL-2 and IFN-α in mouse models of renal cell cancer as IL-2 and IFN-α only induced growth retardation, while IL-12 actually induced tumor regression [Brunda et al, 1995b]

Outline of the thesis

IL-12 has a number of immunoregulatory properties indicating its therapeutic potential against cancer. The encouraging anti-tumor effects, observed in a variety of animal tumor models, have stimulated the development of IL-12 as a single agent for systemic cytokine therapy of cancer in humans. Metastatic renal cell cancer is one of the few human cancers that are more responsive to immunotherapy than to conventional cytotoxic therapies. Therefore, a phase I study of IL-12 was performed in patients with advanced renal cell cancer. The choice of schedule and route of administration were based on experiments in cynomolgus monkeys. IL-12 in s.c. doses of 0.1 to 1.0 μg /kg /day, three times a week, was shown to modulate immune activity without provoking substantial toxicity in these animals.

The objective of the study described in chapter 2 was to evaluate the safety and tolerability of subcutaneous IL-12 in humans and establish the pharmacokinetic profile. The observation of a non-linear relationship between dose and drug exposure in animal models formed the rationale to study the effects of a single and multiple doses of IL-12. In chapter 3 the immunomodulatory activities of IL-12 in humans are described in detail, with emphasis on the induction of secondary cytokines and the effects on circulating leucocyte subset counts. Based on the observation that side effects decreased upon repeated injections of IL-12, we specifically studied whether or not immunomodulatory effects were downregulated in the course of multiple IL-12 injections with special attention for the role of the immunosuppressive cytokine IL-10.

Chapter 4 describes a study of the effect of IL-12 on fibrinolysis and coagulation in humans. This study was performed because several bleeding episodes were reported in simultaneously performed clinical studies, whereas studies in mice and non-human primates had shown that IL-12 induced activation of coagulation and fibrinolysis.

IL-12 is a strong pro-inflammatory cytokine. Studies in patients and experimental animals have demonstrated that endogenously produced IL-12 plays an important role in the toxic sequel of sepsis and endotoxemia. In these situations, excessive activation of various components of the inflammatory cascade contributes to the development of tissue injury and mortality. In chapter 5 we describe the in-vivo effects of different doses of subcutaneous IL-12 on components of the inflammatory cascade. We specifically addressed the

degranulation of neutrophils and the formation of secretory phospholipase A_2, a regulatory enzyme in the formation of eicosanoids.

The study described in chapter 2 was one of four phase I studies, that were simultaneously performed in Europe and the US. Subsequent phase II studies in patients with advanced renal cell cancer and ovarian cancer demonstrated disappointing anti-tumor effects. The results described in chapter 3, together with other pharmacodynamic studies, indicate that the lack of efficacy was accompanied by, and probably related to, declining biological effects of IL-12 in the course of repeated administrations at doses approaching the maximum tolerated dose (MTD). Nevertheless, IL-12 remains a promising immunotherapeutic agent because recent cancer vaccination studies in animal models and humans have demonstrated its powerful adjuvant properties. Chapter 6 reviews the adjuvant properties of IL-12 and delineates how the immunoregulatory properties of IL-12 described in the previous chapters may contribute to the adjuvant effects. In addition, it is discussed how the studies presented in this paper, together with other clinical studies of systemic IL-12, indicate that IL-12 may exert optimal adjuvant effects only at low dose levels. Finally, the future perspectives of IL-12 in the treatment of cancer are addressed.

Chapter 2

A phase I study of subcutaneously administered recombinant human IL-12 in patients with advanced renal cell cancer

Adapted from:

Phase I study of subcutaneously administered recombinant human interleukin 12 in patients with advanced renal cell cancer

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Abstract

A phase I study was conducted to characterize the maximum tolerated dose (MTD), dose limiting toxicity (DLT) and pharmacokinetics of a single dose followed by three times weekly subcutaneous (s.c.) injections of recombinant human interleukin 12 (rHuIL-12).

The study encompassed 28 patients with advanced renal cell carcinoma. RHuIL-12 was administered on day 1, followed by an observation period of 7 days. Starting on day 8, repeated s.c. injections were administered 3 times a week, for 2 weeks.

The MTD of the initial injection was evaluated at dose levels of 0.1, 0.5 and 1.0 μg/kg. DLT was observed at 1.0 μg/kg and consisted of fever, perivasculitis of the skin and leucopenia. The MTD of the subsequent repeated injections after 1 week of rest was studied at dose levels 0.5, 1.0 and 1.25 μg/kg. DLT at 1.25 μg/kg comprised deterioration of performance status, fever, vomiting, mental depression and leucopenia. Other notable toxicities were oral mucositis and elevation of hepatic enzymes. Fever, leucopenia and elevation of hepatic enzymes were more severe after the initial injection than after repeated injections at the same dose level.

At dose level 0.5 μg/kg, the mean area under the plasma concentration-time curve decreased from 7.4 ng x h/ml after the first injection to 3.3 ng x h/ml ($p=0.034$) after repeated administrations, and at dose level 1.0 μg/kg, from 31.8 ng x h/ml to 6.0 ng x h/ml ($p=0.041$). One patient had a partial response and seven patients had stable disease.

Conclusion: The MTD of a single s.c. injection of rHuIL-12 was 0.5 μg/kg and the MTD of 3 subsequent administrations per week was 1.0 μg/kg. In comparison with a single administration, the 3 times weekly administrations at the same dose level were accompanied with a milder pattern of side effects and a reduction of the area under the plasma concentration-time curve.

Introduction

Interleukin 12 (IL-12) is a heterodimeric cytokine with immunoregulatory functions. It stimulates the proliferation and activation of T lymphocytes and natural killer cells [Kobayashi et al, 1989; Perussia et al, 1992] and induces the production of IFN-γ by these cells [Kobayashi et al, 1989; Chan et al, 1991]. IL-12 promotes T- helper type 1 responses [Manetti et al, 1993; Wu et al, 1993], including the commitment of naive helper T cells to the T- helper type 1 developmental pathway, while inhibiting T-helper type 2 development and function, thereby promoting cellular immunity [Scott, 1993]. Additionally, IL-12 can inhibit tumor-associated angiogenesis [Voest et al, 1995; Sgadari et al, 1996]. The antitumor effects of IL-12 have been evaluated in a large number of murine tumor models and more recently in nonhuman primate tumor models [Brunda et al, 1993; Nastala et al, 1994; Fujiwara et al, 1996; Sarmiento et al, 1994; Bree et al, 1994]. Cures and long term survival were seen in murine renal cell carcinoma (RCC) at doses that resulted in mild toxicities. These results show that IL-12 has important immunomodulatory and anti-tumor effects in animal models. Clinical experience with IL-12 in humans is limited. Metastatic RCC appears to be more responsive to immunomodulatory treatment than to conventional cytotoxic chemotherapy. However, despite progress in treatment, overall prognosis remains poor and effective immunotherapies are needed [Motzer et al, 1996]. Therefore, we performed a Phase I study of subcutaneous (s.c.) administration of recombinant human interleukin 12 (rHuIL-12) in patients with RCC.

The choice of schedule and route of administration were based on experiments in cynomolgus monkeys (Hoffmann La Roche, personal communication). IL-12 in s.c. doses of 0.1 to 1.0 μg/kg per day, three times a week, were shown to modulate immune activity without provoking substantial toxicity in these animals. The observation of a nonlinear relationship between dose and drug exposure formed the rationale to study the effects of a single and multiple doses of IL-12. The purpose of the study was to investigate the toxicity and pharmacokinetics of a single s.c. administration of rHuIL-12 and of a schedule of 3 s.c. administrations per week for 2 weeks, started one week after this single s.c. injection.

Patients and Methods

Patient selection

Patients had histologic proof of RCC with measurable locally advanced or metastatic disease. Patients were between 18 and 75 years of age, had a WHO performance score of 0 to 1, a life expectancy > 4 months, adequate renal function (serum creatinine < 1.5 times normal), adequate hepatic function (normal serum bilirubine, serum ALT and/or aspartate-aminotransferase < 2.5 times normal, serum alkaline phosphatase < 2.5 times normal), normal serum calcium, serum hemoglobin > 10 g/dl, white blood count > $3x10^9$/liter, granulocytes > $2x10^9$/liter, platelets > $75x10^9$/liter and normal pulmonary function. They had not received more than one previous immunotherapy. All former therapies were ended at least 6 weeks prior to start of treatment with rHuIL-12. Patients did not use systemic corticosteroids. Patients with brain or leptomeningeal metastases or major fluid effusions (e.g. ascites, pleural effusions) were excluded. Patients with major concurrent systemic disease, an organ graft or a prior history of other malignancy were excluded as were patients known to be seropositive for HIV or Hepatitis B surface antigen. All patients gave written informed consent.

rHuIL-12

rHuIL-12 (Ro 24-7472) was supplied by Hoffmann La Roche, Nutley, NJ, U.S.A and administered by s.c. injection. The first injection was an in-hospital treatment. Subsequent injections were given on an outpatient basis.

Study design

The study was an open label non-randomized Phase I dose escalation trial carried out in two European cancer centers to evaluate the safety and tolerability of an initial single injection of rHuIL-12 as well as the safety and tolerability of repeated s.c. injections administered in treatment cycles of 2 weeks with three injections per week. Pharmacokinetics and pharmacodynamics were studied simultaneously. The treatment protocol was approved by the ethics board of the participating institutions.

Treatment schedule and follow up

On day 1 a single s.c. injection of rHuIL-12 was given, followed by an observation period of 7 days. Subsequently, on day 8 repeated injections were started, with rHuIL-12 s.c., three times a week, for 2 weeks. After a 2-week rest period repeated injections were resumed with an identical schedule. Tumor

volume was assessed after 2 months of treatment. Patients who did not experience tumor progression or DLT could be treated with additional cycles. The dose of rHuIL-12 was calculated per kilogram (kg) body-weight, where 80 kg was taken as the maximum multiplication factor.

The MTD was defined as one dose level below the dose that causes DLT, i.e. the dose that causes drug-related grade 3 or 4 toxicity, with the exception of lymphopenia, in one third of patients. Dose escalation for the initial single injection was decided upon the toxicity encountered during the week of observation that followed, until repeated dosing was started from day 8 onwards. Toxicity was assessed using the National Cancer Institute Common Toxicity Criteria. To define the MTD, cohorts of three patients were entered at each dose level until a grade 3 or 4 toxicity occurred; for these and subsequent dose escalation levels, three more patients were entered. If more than one patient experienced drug-related grade 3 or 4 toxicity, three more patients were entered at the previous dose level. As long as the MTD for the initial single injection had not been reached, the dose of rHuIL-12 for repetitive administrations was identical with the initial dose.

Once the MTD was reached for the initial single dose, this dose was fixed in all subsequent patients. To define the MTD for repeated injections, the toxicity encountered during the first two cycles (8th until 64th day) were evaluated. Further dose escalation steps were carried out as previously described.

Before start of the study, all considered patients underwent a complete medical history and physical examination, electrocardiography, hematology and blood chemistry tests, dipstick urinalysis, pulmonary function test with carbon monoxide (CO) diffusion capacity and measurements of study parameters by chest X-ray and Computerized tomography scan.

After the single initial injection, patients had physical examination and complete blood counts daily and serum chemistry and urinalysis were repeated on days 1 and 2. During the repetitive injections, vital signs and complete blood counts were assessed after all drug administrations and serum chemistry and urinalysis were repeated twice a week. Pulmonary function tests were performed on days 12, 19 and 26. Study parameters were measured by chest X-ray and Computerized tomography scan after each treatment period of 2 months.

Pharmacokinetic sampling and data analysis

Pharmacokinetic parameters were calculated from serum concentrations of rHuIL-12 in blood samples taken before and 4, 6, 8, 10, 12, 18, 24, 30, 36, 48, 72 and 96 hours after the single initial injection. During the repetitive injections,

blood samples were taken on day 15 and 17 prior to drug administration, on day 19, prior to and 4, 6, 8, 10, 12, 18, 24, and 36 hours after drug administration and on day 26.

Serum concentrations of rHuIL-12 were measured by a method of antibody capture followed by a cell proliferation assay [Motzer et al, 1998]. This assay has a lower limit of detection of 50 pg/ml.

Individual patients' plasma concentration-time data were analysed using the Siphar software package (version 4.0; SIMED, Créteil, France) by noncompartmental analysis. The area under the plasma concentration-time curve (AUC) for rHuIL-12 was calculated by the linear trapezoidal rule up to the last sampling point with detectable levels (C), with extrapolation to infinity ($AUC_{0\rightarrow Indefinite}$) by the equation AUC + C/k_{el} , where k_{el} represents the terminal disposition rate constant. The latter term was calculated from the slope of the data points in the final log-linear part of the concentration-time curve by weighed (1/y) least-squares linear regression analysis. Maximum plasma concentrations (C_{max}) and the time to maximum concentration (T_{max}) were estimated by visual inspection of the semilogarithmic plot of the concentration-time curve. The terminal disposition half-life ($t_{½}$) was calculated by dividing ln2 by k_{el} .

To test parameter differences for statistical significance among treatment courses, a two-tailed paired Student's t-test was performed. Probability values of less than 0.05 were regarded as statistically significant. All statistical calculations were performed using Number Cruncher Statistical System (NCSS, version 5.X; Dr. Jerry Hintze, Kayesville, UT, USA) and STATGRAPHICS Plus (version 2; Manugistics Inc., Rockville, MD, USA).

Results

Patient population

The characteristics of the 28 patients who participated in the study are given in Table 1. Twenty patients were male. The median age was 56 years. Twenty-seven patients had undergone nephrectomy and 13 of them received surgical treatment only. There were nine patients pretreated with immunotherapy (IFN-γ, IL-2 or a combination of IFN-α and IL-2 with or without lymphokine-activated killer cells), two patients with chemotherapy and four patients with immunotherapy and chemotherapy. The results of three patients were excluded from analysis: One patient erroneously received an overdose at the first injection without major sequela. In two patients, both with a history of

atrial fibrillation, this arrhythmia recurred after the first single injection. These patients were removed from the study. Twelve patients received one treatment cycle, nine patients received two cycles and four patients had more than two cycles with a maximum of six.

Table 1. Patient characteristics

Number of patients		28	
Median age (years)		56	(41-70[¥])
Gender (male/female)		20/8	
Performance status			
Karnofsky	100%	15	
	90%	9	
	80%	4	
Previous therapy			
Surgical only		13	
Immunotherapy		9	
Chemotherapy		2	
Chemo- and immunotherapy		4	
Median duration of disease (months)		38	(1-264)
Disease status			
Locally advanced		1	
Metastatic		27	

¥ Results are given in numbers; a range is shown in parentheses.

Side effects and laboratory abnormalities

Common side effects and laboratory abnormalities observed after the first, as well as after repeated injections of rHuIL-12, were fever and flu-like symptoms (chills, sweating, headache, myalgia), anorexia, nausea, vomiting, fatigue, leucopenia, lymphopenia, granulopenia, anemia, thrombopenia, hypocalcemia and elevation of hepatic enzymes. Oral mucositis, reduction of pulmonary CO diffusion capacity and hyponatremia mainly occurred after repeated injections. Fever sometimes persisted for several days after discontinuation of rHuIL-12.

Table 2. Toxicity profile initial single injection (part 1)

Dose level	*Hemoglobin*				*Leukopenia*				*Granulopenia*				*Lymphopenia*				*Thrombopenia*			
(no of patients)	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0.1 μg/kg (3)	0	0	0	0	1	1	0	0	1	0	0	0	0	2	1	0	0	0	0	0
0.5 μg/kg (6)	3	0	0	0	0	1	1	0	0	1	0	0	1	5	0	0	0	0	0	0
1.0 μg/kg (4)	3	0	0	0	0	3	1	0	1	2	0	0	1	2	0	1	4	0	0	0
0.5 μg/kg (12)*	2	0	0	0	3	2	3	0	3	2	3	0	3	5	2	1	2	0	0	0

*After defining MTD for the initial injection, the additional patients enrolled to define MTD for repeated injections received their initial injection at a dose of 0.5 μg/kg

Table 3. Toxicity profile initial single injection (part 2)

Dose level	*Fever*				*Fatigue*				*Headache*				*Nausea*				*Vomiting*			
(no of patients)	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0.1 μg/kg (3)	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
0.5 μg/kg (6)	1	5	0	0	2	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
1.0 μg/kg (4)	0	3	1	0	1	3	0	0	2	0	0	0	3	0	0	0	2	0	0	0
0.5 μg/kg (12)*	3	9	0	0	1	1	0	0	6	0	0	0	4	0	0	0	2	1	0	0

*After defining MTD for the initial injection, the additional patients enrolled to define MTD for repeated injections received their initial injection at a dose of 0.5 μg/kg

Table 4. Toxicity profile initial single injection (part 3)

Dose level	*Mucositis*			*Skin*			*Diarrhea*			*Pulmonary*			*Liver enzyems*			*Hypo-natremia*		
(no of patients)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
0.1 μg/kg (3)	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
0.5 μg/kg (6)	0	0	0	0	0	0	0	0	0	0	0	0	3	1	0	2	0	0
1.0 μg/kg (4)	0	1	0	0	0	1	0	0	0	0	0	0	2	2	0	0	0	0
0.5 μg/kg (12)*	0	0	0	0	0	0	0	1	0	0	0	0	2	1	0	0	2	0

*After defining MTD for the initial injection, the additional patients enrolled to define MTD for repeated injections received their initial injection at a dose of 0.5 μg/kg

Determination of the MTD for the single initial dose

The dose levels studied for the initial single dose were: 0.1 μg/kg, 0.5 μg/kg and 1.0 μg/kg. Tables 2-4 present the side effects and their grading at these dose levels.

Three patients received a single initial rHuIL-12 dose of 0.1 μg/kg. No grade 3 toxicities were observed. On dose level 0.5 μg/kg, six patients were treated and one developed a grade 3 leucopenia.

Of the four patients who received 1.0 μg/kg, three developed DLT. One patient had a grade 3 leucopenia with grade 3 fever and one experienced a grade 2 leukopenia that lasted 10 days, necessitating delay of the repetitive injection cycle. The third patient developed an erythema of the skin, which persisted for 8 days. Histology of the lesions showed a perivasculitis. Consequently, 0.5 μg/kg of rHuIL-12 was regarded as MTD for the initial single dose.

Therefore, the 12 patients, who were subsequently enrolled to define the MTD of repetitive injections, had their first injections fixed at a dose of 0.5 μg/kg. The side effects of these 12 patients are displayed in the bottom lines of Table 2. Of note, three of 12 patients experienced grade 3 leuco- and granulopenia.

Determination of the MTD for the repetitive administration cycles

The dose levels studied for the repetitive administration cycles were: 0.5 μg/kg, 1.0 μg/kg and 1.25 μg/kg. In Tables 5-7, the toxicities according to these dose levels are shown. The worst observed toxicities observed in the first two treatment cycles were analysed.

Six patients, who had received a single initial dose of rHuIL-12 at 0.5 μg/kg received the same dose from day 8 onwards, three times a week. Because three of the 12 additional patients at the initial single dose of 0.5 μg/kg experienced grade 3 leuko- and granulopenia, we decided to extend our experience at this dose level with repeated injections. Hence, nine patients received repeated injections, three times per week, at 0.5 μg/kg. Only one developed a grade 3 granulopenia during repeated administrations. These nine patients had a reduction of certain side effects and laboratory abnormalities (fever, leucopenia and elevation of hepatic enzymes) when the single initial injection was compared with the repeated injections (tables 5-7). This effect was also observed at consecutive higher dose levels. The next three patients received repeated injections at 1.0 μg/kg without grade 3 or 4 toxicities. However, at a dose of 1.25 μg/kg, four of four patients exhibited DLT: All had grade 3 progressive fatigue with deterioration of performance status.

Table 5. Toxicity profile repeated injections, worst of cycles 1 and 2 (part 1)

Dose level	*Hemoglobin*				*Leukopenia*				*Granulopenia*				*Lymphopenia*				*Thrombopenia*			
(no of patients)	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0.5 µg/kg (9)	3	0	0	0	5	3	0	0	3	2	1	0	4	3	1	0	0	0	0	0
1.0 µg/kg (5)	2	0	0	0	2	1	0	0	3	0	0	0	0	5	0	0	0	0	0	0
1.25 µg/kg (4)	4	0	0	0	1	1	1	0	1	2	0	0	0	2	2	0	0	1	0	0

Table 6. Toxicity profile repeated injections, worst of cycles 1 and 2 (part 2)

Dose level	*Fever*				*Fatigue*				*Headache*				*Nausea*				*Vomiting*			
(no of patients)	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0.5 µg/kg (9)	3	4	0	0	5	0	0	0	3	1	0	0	4	0	0	0	4	0	0	0
1.0 µg/kg (5)	0	3	0	0	2	3	0	0	1	0	0	0	1	1	0	0	1	1	0	0
1.25 µg/kg (4)	0	2	0	1	0	0	4	0	1	1	0	0	1	1	1	0	1	1	1	0

Table 7. Toxicity profile repeated injections, worst of cycles 1 and 2 (part 3)

Dose level	*Mucositis*				*Pulmonary*				*Liver enzymes*				*Hyponatremia*				*Hypocalcemia*			
(no of patients)	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0.5 µg/kg (9)	2	1	0	0	1	0	0	0	2	0	0	0	4	1	0	0	1	0	0	0
1.0 µg/kg (5)	2	1	0	0	2	1	0	0	4	0	0	0	2	1	0	0	1	1	0	0
1.25 µg/kg (4)	2	2	0	0	1	1	0	0	2	1	0	0	2	2	0	0	1	0	0	0

One patient had grade 3 leucopenia and grade 4 fever, one patient had grade 3 vomiting and nausea and one patient developed grade 3 psycho-neurotoxicity (a mental depression persisting for weeks after discontinuation of rHuIL-12). As indicated in the protocol, more patients were subsequently entered at the previous dose level of 1.0 μg/kg. In two additional patients no grade 3 or 4 toxicity was observed. Added to the 3 patients earlier enrolled on this dose level, a total of five patients were treated with repeated injections at 1.0 μg/kg.

Because none suffered grade 3 or 4 adverse event, we declined from entering a sixth patient. Consequently, 1.0 μg/kg of rHuIL-12 was regarded as MTD for the repeated injections.

Unrelated side effects

Five patients developed complications unrelated to the study medication. In a patient that died of ventricular fibrillation, 11 days after the last rHuIL-12 administration, autopsy showed extensive coronary atherosclerosis.

A patient with a history of myocardial infarction had congestive heart failure. Of two patients with retroperitoneal lymphnode metastasis, one developed hydronephrosis and renal insufficiency and the other developed extensive deep venous thrombosis. Finally, a patient had gastrointestinal bleeding, due to duodenal tumor invasion.

Tumor response

Tumor response could be evaluated in 22 patients. A partial response, which lasted 4 months, was observed in a patient treated with repeated injections at dose level 0.5 μg/kg. At study entry, the patient had local tumor recurrence and bone metastasis, 5 months after diagnosis of RCC and nephrectomy. He had not received prior systemic treatment. Seven Patients had stable disease that lasted between 2 and 6 months. All other patients had progressive disease. Sudden death and cardiac failure impeded evaluation of response in two patients and in another patient who only received a first dose of IL-12, evaluation was omitted.

Pharmacokinetics

Results of the pharmacokinetic studies are shown in Table 8. Samples for pharmacokinetic analysis, after the initial injection and subsequent injections, could be obtained from all 13 patients that were enrolled to define the MTD for the initial injection. From 6 additional patients, entered to define the MTD of repeated injections, samples after the initial injection at 0.5 μg/kg could be analysed. At the 0.1 μg/kg dose level, serum concentrations of rHuIL-12 were

below the assay's detection limit, after the initial single injection as well as after repeated injections. After an initial injection of 0.5 µg/kg, serum rHuIL-12 increased to a mean level of 362 pg/ml (C_{max}). Peak levels of rHuIL-12 were reached at a mean of 9.67 hours (T_{max}) after administration.

Table 8. Pharmacokinetic data

Mean (± SD) pharmacokinetic parameters at dose levels 0,5 µg/kg and 1.0 µg/kg on days 1 and 19

		0.5 / 0.5		**1.0 / 1.0**	
	Unit	Day 1	Day 19	Day 1	Day 19
		n = 12	n = 4	n = 4	n = 2
Tmax	h	9.7 (5)	9.5 (2.2)	17 (7)	4 (0)
T ½	h	9.4 (3.5)	7.9 (1.6)	12.1 (3.6)	4.9 (-)¥
Cmax	pg/ml	362 (214)	255 (200)	1131 (1051)	376 (49)
AUC	ng x t/ml	7.4 (5.4)*	3.3 (1.6)*	31.8 (22.3) #	6 (2.4)#

* Pvalue: 0.034
\# Pvalue: 0.041
¥ n = 1

C_{max}: maximum plasma concentration
T_{max}: time to maximum concentration
AUC: Area under the plasma concentration-time curve extrapolated to infinity
$T_{½}$: terminal disposition half life

The mean half life ($T_{½}$) of rHuIL-12 was 9.36 hours. Mean C_{max}, T_{max} and $T_{½}$ for repeated administrations at the 0,5 µg/kg dose level did not differ significantly from the results after the initial injection. However, after repeated dosing of rHuIL-12, a considerable decrease of 55% in serum AUC was observed. The mean AUC of 7.43 ng x h/ml after the initial injection dropped to a mean AUC of 3.33 ng x h/ml after the last injection of the first cycle of repeated administration. This difference was highly significant (p=0.034). After an initial injection of 1.0 µg/kg, mean C_{max} was 1131 pg/ml, with a mean T_{max} of 17 hours and a mean $T_{½}$ of 12.1 hours. These values did not differ significantly when measured after repeated injections. However, at this dose level, a significant decrease of 80% in serum AUC could be demonstrated as well, when comparing

the AUC after repeated injections with the AUC after the initial injection (p=0.041). In none of the patients could the development of antibodies to rHu-IL-12 be detected during treatment.

Discussion

The primary objective of this Phase I study was to define the MTD of an initial single s.c. administration of rHuIL12 and of subsequent repeated doses and to study pharmacokinetics. The MTD of the initial single injection was 0.5 μg/kg, whereas the MTD of the repeated administrations was 1.0 μg/kg. DLTs of the single injection consisted of fever, perivasculitis of the skin and leucopenia. DLTs of repeated injections were progressive fatigue and deterioration of performance status, mental depression, nausea, vomiting and leucopenia.

The MTDs are comparable with those reported in other Phase I studies [Motzer et al, 1998; Atkins et al, 1997]. In a trial that compared a fixed, s.c., once a week dose scheme with an up-titration schedule of IL-12, the MTD's were 1.0 and 1.5 μg/kg, respectively and DLTs were elevation of transaminase concentration, pulmonary toxicity and leucopenia [Motzer et al, 1998]. In another trial, with IL-12 administered by i.v. injection for 5 days a week, after an initial injection 2 weeks earlier, DLT consisted of liver function abnormalities and oral mucositis and MTD was 0.5 μg/kg [Atkins et al, 1997]. In contrast, we did not encounter hepatotoxicity as a dose limiting adverse event.

The toxicity profile that we observed resembles that encountered in other studies [Motzer et al, 1998; Atkins et al, 1997; Bajetta et al, 1998], but two patients suffered side effects not earlier described in association with IL-12 therapy. One patient, who had no psychiatric history, experienced mental depression, necessitating antidepressive medication. Although similar complaints have not been attributed to treatment with IL-12 thusfar, the use of other cytokines, such as IL-2 [Denicoff et al, 1987] and IFN-α [Renault et al, 1987], has induced mental depression and a variety of other neuropsychiatric complaints. Another patient developed erythema of the skin and histological examination showed a perivasculitis. In a pilot study at 0.5 μg/kg, a skin rash was observed in 2 patients, for whom no biopsies were available [Bajetta et al, 1998]. Extreme progressive fatigue with severe deterioration of performance status was dose limiting for all patients treated at 1.25 μg/kg. Fatigue was not described as dose limiting in other Phase I studies. It was however a consistent observation in murine and primate models [Bree et al, 1994; Coughlin et al, 1997].

Furthermore, in an early terminated Phase II study, one third of patients suffered grade 3 to 4 fatigue [Leonard et al, 1997].

We observed a reduction of 55 to 80% of the mean AUC when repeated injections of IL-12 were compared with the initial injection at the same dose level. This was accompanied by reduction of side effects and resultant increase of the MTD. Motzer [Motzer et al, 1998] observed an AUC after up-titration dose escalation that was lower than the AUC of the first injection at the same dose. We could not attribute the decrease of the AUC with repeated injections to inhibited resorption or greater clearance from the peripheral blood of free IL-12, because T_{max} and $T_{½}$ did not change significantly. Neither did we observe the development of IL-12 antibodies. However, a possible explanation, which we have not investigated, would be that concentrations of soluble IL-12 receptors increase in the course of IL-12 treatment. IL-12 has been shown to upregulate its own receptors in peripheral blood CD56+ NK cells [Naume et al, 1993a]. Also, IL-12 enhances the expression of mRNA transcripts of one of the subunits of the IL-12 receptor in naive T-cells [Rogge et al, 1997]. For other cytokines, a negative feedback mechanism operates at persistent high cytokine levels, by the increased release of soluble cytokine receptor fragments that inhibit the effects of the cytokines. The eventual existence of such a mechanism in IL-12 requires additional research.

Recently, it was shown in humans that the insertion of a treatment free period of a week after the first administration of IL-12 reduces the toxicity of subsequent injections [Leonard et al, 1997; Cohen, 1995]. In a study that used a dose of IL-12 that was previously well tolerated in a schedule that was identical except for the omission of a treatment free period after the first dose, severe toxicity and deaths occurred and necessitated early cessation of the study [Atkins et al, 1997; Leonard et al, 1997]. Subsequently it was shown in murine and primate models that a single injection of IL-12 before consecutive daily dosing protected the animals from toxicity and mortality and was accompanied by reduced IFN-γ levels [Coughlin et al, 1997; Leonard et al, 1997; Sacco et al, 1997]. Reduced IFN-γ production seems an important feature of the down-regulation of the toxic effects of IL-12 in the course of treatment [Sacco et al, 1997]. Many of the side effects that accompany IL-12 therapy are considered to be IFN-γ dependent because they are also encountered in studies with IFN-γ [Quesada, 1995; Thompson et al, 1987]. In a pilot study of s.c. IL-12, 0.5 μg/kg/week, a decrease of IFN-γ peak levels was demonstrated with subsequent injections [Bajetta et al, 1998]. Although Atkins et al report higher IFN-γ peak levels during the first 5-day treatment course than after the initial dose, peak

levels thereafter declined during subsequent cycles [Atkins et al, 1997]. IL-10 may be another cytokine that contributes to the counter regulation of the biological effects of IL-12. We have observed a decrease of IFN-γ peak levels together with increased levels of IL-10 in the course of treatment [Portielje et al, 2002a].

Our results raise the question whether the anti-tumor activity of IL-12 is downregulated in the course of treatment as well. An unfortunate correlation may exist between IL-12 induced toxicity and anti-tumor efficacy.

Antibody neutralization of endogeneous IFN-γ was shown to suppress antitumor effectiveness of IL-12 in murine models. Therefore, part of the anti-tumor effects of IL-12 seems due to induction of IFN-γ. However, exogeneous administration of IFN-γ is not as effective as IL-12 [Nastala et al, 1994; Fujiwara et al, 1996; Coughlin et al, 1995]. Additionally, it was shown in an animal model that giving a single dose of IL-12, a week prior to daily administration, did diminish IFN-γ induction and toxic effects but left the anti-tumor activity largely unaffected [Coughlin et al, 1997].

In conclusion, the s.c. administration of rHuIL-12 as an initial injection at a dose of 0.5 μg/kg, followed after a week rest by repeated injections at a dose of 1.0 μg/kg, 3 times a week, was well tolerated. A decrease of toxicities and a reduction in the AUC of IL-12 was observed with repeated injections.

Chapter 3

Repeated administrations of IL-12 are associated with persistently elevated plasma levels of IL-10 and declining IFN-γ, TNF-α, IL-6 and IL-8 responses

Adapted from:

Repeated administrations of IL-12 are associated with persistently elevated serum levels of IL-10 and declining IFN-γ, TNF-α, IL-6 and IL-8 responses

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Abstract

Repeated administrations of recombinant human IL-12 (rHuIL-12) to cancer patients is characterized by a reduction of side effects during treatment. Induction of IFN-γ, considered a key mediator of anti-tumor effects of IL-12, is known to decline upon repeated administrations. We studied whether other immunological effects of rHuIL-12 are tapered in the course of treatment.

In a phase I study of 26 patients with advanced renal cell cancer, rHuIL-12 was administered s.c. on day 1, followed by 7 days rest and 6 injections administered over a 2-week time period. Plasma concentrations of various cytokines were monitored, as well as absolute counts of circulating leukocyte and lymphocyte subsets.

The first injection of IL-12 was accompanied by rapid, transient and dose dependent increments of plasma levels IFN-γ, TNF-α, IL-10, IL-6, IL-8, but not IL-4, as well as rapid, transient and dose dependent reductions of lymphocyte, monocyte and neutrophil counts. The major lymphocyte subsets, i.e., CD4+ and CD8+ T cells, B cells and NK cells, followed this pattern. Upon repeated rHuIL-12 injections, IL-10 concentrations increased further, whilst the transient increments of IFN-γ, TNF-α, IL-6 and IL-8 concentrations, as well as the fluctuations of the leukocyte subset counts were tapered. Dose escalation of IL-12 within clinically tolerable margins did not reduce the decline of these immunological effects.

Conclusion: Induction of pro-inflammatory cytokines and associated fluctuations in leucocyte subset counts decrease upon repeated administrations of rHuIL-12. The steady increment of IL-10 plasma levels may mediate the observed down-regulation of clinical and immunological effects.

Introduction

Interleukin 12 (IL-12) is a cytokine with an important function in the regulation of the cell-mediated immune response. In animal and *in vitro* models, IL-12 stimulates CD4+ Th-1 responses and promotes the proliferation and activation of natural killer (NK) cells and stimulates them to produce interferon-gamma (IFN-γ) [Trinchieri, 1995]. IL-12 also stimulates antigen specific CD8+ T cell responses [Mortarini et al, 2000; Curtsinger et al, 1999]. Additionally, IL-12 has anti-angiogenic properties [Gee et al, 1999] and a direct growth inhibitory effect on tumor cells [Su et al, 2001]. The promising anti-tumor effects that were observed in murine and non-human primate models [Brunda et al, 1993] prompted clinical studies in patients with cancer. Efficacy studies of recombinant human IL-12 (rHuIL-12) have now been performed in patients with ovarian cancer and renal cell cancer, but anti-tumor responses have been disappointing [Motzer et al, 2001; Hurteau et al, 2001]. During Phase I testing of recombinant human IL-12, we and others have observed a decrease of side effects upon repeated administrations in conjunction with a reduction of IFN-γ release [Portielje et al, 1999; Coughlin et al, 1997; Leonard et al, 1997; Sacco et al, 1997;]. Because IFN-γ is considered to be a key mediator of anti-tumor effects of IL-12 [Fujiwara et al; 1997], the down-regulation of IFN-γ release observed upon repeated IL-12 administrations may be related to the lack of anti-tumor effects of IL-12.

Here we assessed whether or not the down-regulation of IFN-γ production that occurs upon repeated rHuIL-12 administrations: (a) comprised other immunological effects, (b) was accompanied by up-regulation of an inhibitory immune regulatory mechanism or cytokine, and (c) could be prevented by dose escalation of rHuIL-12. To this end, we studied the *in vivo* effects of rHuIL-12 in 26 patients with advanced renal cell cancer, treated in a phase I study [Portielje et al, 1999]. We studied: (a) plasma levels of cytokines: IFN-γ, TNF-α, IL-10, IL-8, IL-6 and IL-4; (b) absolute numbers of circulating neutrophils, lymphocytes and monocytes, and (c) the major lymphocyte subsets: CD4+ and CD8+ T cells, B cells and NK cells. We compared the effects of the first rHuIL-12 dose with effects of subsequent repeated administrations at the same or escalated doses.

Patients and Methods

Study design

We studied 26 patients with locally advanced or metastatic renal cell cancer who participated in an open-label, non-randomized phase I dose escalation trial. This trial was carried out in the Rotterdam and Mainz cancer centers to evaluate the safety and tolerability of an initial single s.c. injection of rHuIL-12, followed by repeated injections. Patients had a median age of 56 years (range 41 to 70 years). They had not received more than one previous immunotherapeutic intervention and all former therapies were terminated at least 6 weeks prior to start of treatment with rHuIL-12. Inclusion criteria were a World Health Organization (WHO) performance score of 0 or 1 and adequate hematological, renal, hepatic, cardiovascular and pulmonary functions. None of the patients received systemic corticosteroid therapy. All patients had given written informed consent.

RHuIL-12 (Ro 24-7472) was supplied by Hoffmann-La Roche (Nutley, N.J.) and administered by s.c. injections. On day 1 a single injection of rHuIL-12 was given, followed by an observation period of 7 days. Subsequently, on day 8, a 2-week cycle was started, with 3 injections per week. Immuno-modulatory effects of the first administration of rHuIL-12 were studied in 26 patients after a dose of 0.1 μg/kg (n=3), 0.5 μg/kg (n=19) or 1.0 μg/kg (n=4). The immunological effects of repeated injections were studied in 18 patients who received all 7 injections each. Twelve of these patients received the same dose of rHuIL-12 for the initial as well as the repeated injections: 0.1 μg/kg (n=3), 0.5 μg/kg (n=7) or 1.0 μg/kg (n=2). The remaining 6 patients started with an initial dose of 0.5 μg/kg rHuIL-12 and repeated injections were administered at a dose of 1.0 μg/kg (n=4) or 1.25 μg/kg (n=2).

Cytokines

EDTA-anticoagulated venous blood samples for measurement of cytokines were obtained directly before and 4, 8, 12, 24, 48 and 72 hours after the first and seventh administration of rHuIL-12. In 12 patients blood was also obtained 96 and 168 hours after the first injection. Plasma was obtained after centrifugation of blood for 10 minutes at 1300g. Plasma samples were stored at -70°C until tested. Serum concentrations of bio-active IL-12 were measured by a method of antibody capture followed by a cell proliferation assay with a lower limit of detection of 50 pg/ml [Motzer et al, 1998]. IFN-γ, TNF-α, IL-10, IL-8, and IL-6 concentrations were determined with commercially available enzyme

amplified sensitivity immunoassays (Medgenix EASIA, Biosource Europe, Fleurus, Belgium). The lower limits of detection of the assays were: IFN-γ: 0.03 IU/ml; TNF-α: 3 pg/ml; IL-10: 1 pg/ml; IL-8: 1 pg/ml; IL-6: 2 pg/ml and IL-4: 0.2 pg/ml.

Determination of absolute numbers of peripheral-blood leukocyte subsets

Blood samples for determination of absolute numbers of peripheral blood leukocyte subsets were obtained from 22 patients after an initial dose of 0.1 μg/kg (n=3), 0.5 μg/kg (n=15) or 1.0 μg/kg (n=4). Samples were obtained directly before and 1, 2, 3, 4 and 7 days after the first administration of rHuIL-12, before every subsequent administration, and 7 days after the last administration. Leukocyte concentrations and differential counts were determined in EDTA anticoagulated blood samples using a Technicon H1 automated cell counter (Technicon, Tarrytown, NY).

Lymphocyte Immunophenotyping

Immunophenotyping was performed on blood specimens from 9 patients. All patients studied received an initial injection of 0.5 μg/kg rHuIL-12. The repeated injections were dosed at 0.5 μg/kg (n=4), 1.0 μg/kg (n=3) or 1.25 μg/kg (n=2). Heparinized venous blood samples for immunophenotyping were obtained directly before (day 0) and one day after the first administration (day 1), and directly before (day 19) and one week after (day 26) the last administration of rHuIL-12. For immunostaining the erythrocytes were lysed by ammonium-chloride. The remaining leukocytes were washed and stained using the following mixtures of monoclonal antibodies (mAb) conjugated either with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): CD45 FITC + CD14 PE; CD3 FITC + CD16 PE + CD56 PE; CD3 FITC + CD4 PE; CD3 FITC + CD8 PE; CD19 PE. Isotype control mAb (mouse IgG_{2a} FITC, mouse IgG_1 FITC and mouse IgG_1 PE) were used to visualize non-specific antibody binding. The CD8 PE mAb was obtained from DAKO (Glostrup, Denmark). All other mAbs were purchased from BD Biosciences (San Jose, CA). Sample processing and flow cytometry were performed as described elsewhere [Gratama et al, 1996].

Statistical analyses

To test differences for any parameter between paired samples, for statistical significance, two-tailed paired Student's t-tests were performed. To test differences for any parameter between non-paired samples, two-tailed non-

paired Student's t-tests were performed. *P* values <0.05 were considered statistically significant.

Results

Cytokines

The first administration of rHuIL-12 was followed by increments in plasma concentrations of IFN-γ, TNF-α, IL-6, IL-8 and IL-10, whereas virtually no elevation of IL-4 concentrations was observed (Figure 1). Table 1 shows the baseline and peak levels of cytokines as measured in individual patients during 7 days following the first rHuIL-12 injection, stratified by rHuIL-12 dose level. The pharmacokinetics of rHuIL-12 in this study has been reported previously [Portielje et al, 1999]. Samples from 25 patients were available for IL-12 assessments.

After 0.5 μg/kg rHuIL-12, increments of plasma IL-12 were observed in 10 out of 18 patients and after 1.0 μg/kg rHuIL-12, increments were observed in all four patients. At the lowest dose, i.e. 0.1 μg/kg rHuIL-12, plasma concentrations of IL-12 remained undetectable in all 3 patients. After 0.5 μg/kg, IL-12 became detectable in 10 of 18 patients and after 1.0 μg/kg, IL-12 became detectable in all 4 patients.

The initial induction of IFN-γ, TNF-α, IL-6, IL-8 and IL-10 occurred in a rHuIL-12 dose-dependent way. Following administration of the lowest dose, i.e. 0.1 μg/kg, only a significant increment of TNF-α plasma levels was observed. Upon administration of 0.5 μg/kg, peak levels of IFN-γ, TNF-α, IL-6, IL-8 and IL-10 were significantly higher than the corresponding levels prior to therapy. At the highest rHuIL-12 dose level, i.e. 1.0 μg/kg, peak levels of IFN-γ, TNF-α, IL-8 and IL-10 were even higher than those observed after administration of 0.5 μg/kg. Of note, no increments of IL-4 plasma levels were observed at the 0.1 and 0.5 μg/kg dose levels, whilst these became just detectable within 24 h after administration of 1.0 μg/kg rHuIL-12.

The first rHuIL-12 injection resulted in increments of plasma concentrations of IFN-γ and TNF-α in all patients, whereas increments of IL-10 were demonstrated at the 0.1 μg/kg dose level in 1 of 3 patients (33%), at the 0.5 μg/kg dose level in 11 out of 19 patients (58 %), and at the 1.0 μg/kg dose level in all 4 patients (100%). The first rHuIL-12 injection resulted in increments of IL-8 in all but 5 patients, 3 of whom already had elevated baseline levels of IL-8 (between 26 and 58 pg/ml).

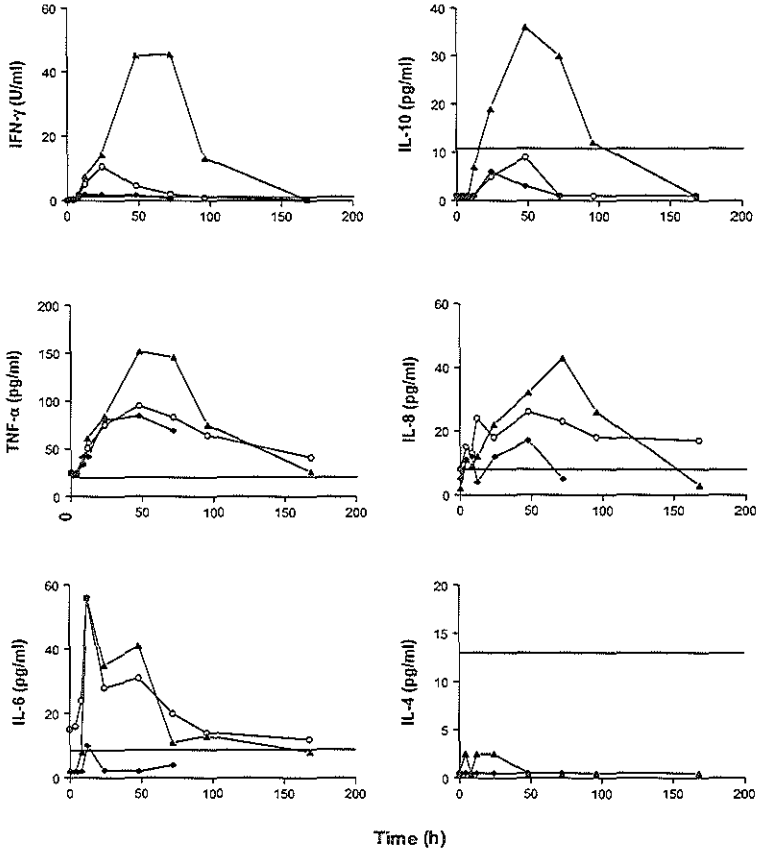

Figure 1. Plasma concentrations of IFN-γ, IL-10, TNF-α, IL-8, IL-6, IL-4 and IL-12 after the first s.c. injection of IL-12.

Median plasma concentrations of IFN-γ, IL-10, TNF-α, IL-8, IL-6 and IL-4 during a period of seven days after the first s.c. administration of 0.1 μg/kg rHuIL-12 (◆, 3 patients), 0.5 μg/kg (O, 19 patients) or 1.0 μg/kg (▲, 4 patients). Dotted lines indicate the upper limit of the normal range.

Table 1. Baseline and maximum cytokine concentrations after the first subcutaneous administration of rHuIL-12

	0.1μg/kg (n=3)				*0.5 μg/kg (n=19)*				*1.0 μg/kg (n=4)*			
	Baseline		Maximum		Baseline		Maximum		Baseline		Maximum	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
IL-12 (pg/ml)	<50	-	<50	-	<50 ¤	-	298 **	116- 830	<50	-	548	475-2630
IFN-γ (IU/ml)	<0.03	<0.03- 0.4	2.6*	1.4 - 2.9	<0.03	<0.03 - 1.4	13 **	1.5 - 41	0.4	<0.03- 0.5	55	12 - 184
IL-10(pg/ml)	<1 #	-	15	-	<1 £	<1 - 6	22 **	4 - 49	<1	-	37 *	27 – 81
TNF-α(pg/ml)	26	21 – 40	84 *	73 - 108	25	8 - 83	95 **	60 - 143	25	7 - 33	168 *	149 - 223
IL-8 (pg/ml)	5	<1 – 15	24	11 - 53	11§	<1 - 35	38 *	14 - 223	< 1	<1 - 8	53 *	32 - 87
IL-6 (pg/ml)	6 ¥	<2 – 10	25	22 - 27	16 ‡	<2 - 79	82 **	17 - 280	< 2	-	83 *	42 - 137

* = p< 0.05, ** = p<0.001; Paired comparison between maximum levels and baseline levels.
As described in the results, induction of the various cytokines did not occur in all patients. In this table only data from patients with increments of cytokine concentrations post IL-12 are shown. Symbols denote the numbers of patients in which increments of cytokine plasma levels have been observed: # n=1; § n= 14; £ n=13; ¥ n=2; ‡ n=16; ¤ n=10.

Increments of IL-6 levels were observed in all but 4 patients, 1 of whom already had a strongly elevated base-line level of 91 pg/ml. IL-6 plasma concentrations were the first to peak at a median of 12 hours post injection (range 8-72 hours in individual patients), followed at 24 hours by IFN-γ (range 12-72 hours) and at 48 hours by TNF-α (range 24-72 hours), IL-10 (range 8-72 hours) and IL-8 (range 8-168 hours) (Figure 1).

Thereafter, levels of TNF-α, IFN-γ, IL-10, IL-8 and IL-6 gradually declined. IFN-γ and IL-10 became undetectable at 7 days after rHuIL-12 injection, whilst TNF-α, IL-6 and IL-8 remained detectable around the upper limits of their normal ranges.

Comparison of the effects of the first and seventh injection of rHuIL-12 on cytokine plasma levels revealed that IL-10 still showed a significant increment after 7 injections at the same dose level (i.e., 0.5 μg/kg; Figure 2, Panels A and B). In contrast, IFN-γ showed only a marginally significant increment after the seventh rHuIL-12 injection (Panels G and H), whilst TNF-α (Panels D and E), IL-8 and IL-6 (data not shown) showed hardly any increments as compared with the corresponding increments after the first injection of the same dose of rHuIL-12.

The reduced increments of plasma levels of these cytokines were not only observed at the 0.5 μg/kg dose level, but also at the 0.1 and 1.0 μg/kg dose levels (results not shown).

The effect of IL-12 dose escalation was further studied in 6 patients who received, after the initial injection of 0.5 μg/kg rHuIL-12, subsequent injections at doses of 1.0 or 1.25 μg/kg rHuIL-12. These dose escalations resulted in an even stronger increment of IL-10 plasma levels as compared to the 0.5 μg/kg dose level (Figure 2, Panels A to C). However, increments of IFN-γ (Panels G to I), TNF-α (Panels D to F), IL-6 and IL-8 plasma levels (data not shown) remained clearly less after the seventh than after the first rHuIL-12 injection.

Leukocyte subsets

The administration of rHuIL-12 was followed by a rapid and transient decrease of absolute numbers of lymphocytes, monocytes and neutrophils in the peripheral blood (figure 3). Table 2 shows the baseline values and nadirs of these cell counts after the first injection of rHuIL-12. The reduction of lymphocytes, monocytes and neutrophils occurred in a rHuIL-12 dose dependent way. The lymphocytes reached their nadir at 1 to 2 days after rHuIL-12 administration, followed by the monocytes at 2 to 3 days, and the neutrophils at 3 to 4 days. At the 0.1 μg/kg dose level, all leucocyte subsets had returned to their baseline levels within 7 days.

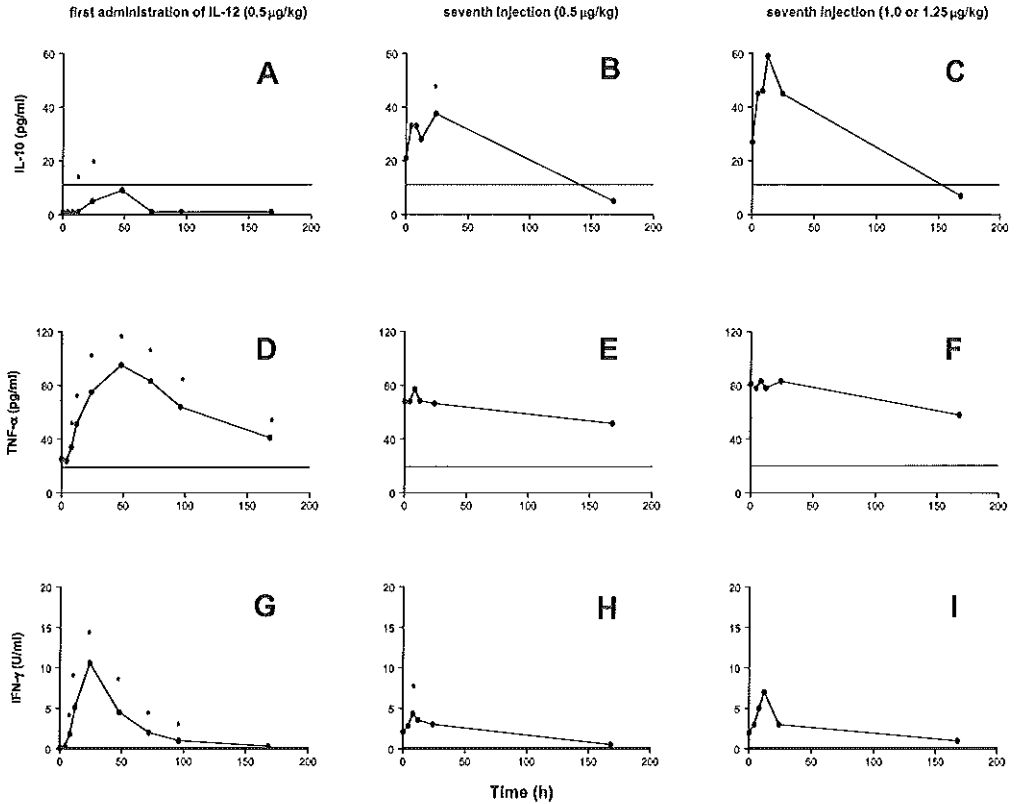

Figure 2. Cytokine concentrations after the first injection of IL-12 (A), compared to concentrations after the seventh injection at the same (B), or escalated dose of IL-12 (C). *Median plasma concentrations of IL-10, TNF-α and IFN-γ were measured during a period of 7 days after the first and after the seventh administration of rHuIL-12. 13 Patients received a first s.c. injection of 0.5 µg/kg (panels A, D and G). Subsequently, 7 of them received, starting on day 8, repeated administrations at the same dose level (panels B, E and H) and 6 of them received subsequent administrations at dose levels 1.0 or 1.25 µg/kg rHuIL-12 (panel C, F and I). Significant differences between concentrations before and after IL-12 injection are indicated by an asterix ($p < 0.05$, paired samples). Dotted lines indicate the upper limit of the normal range.*

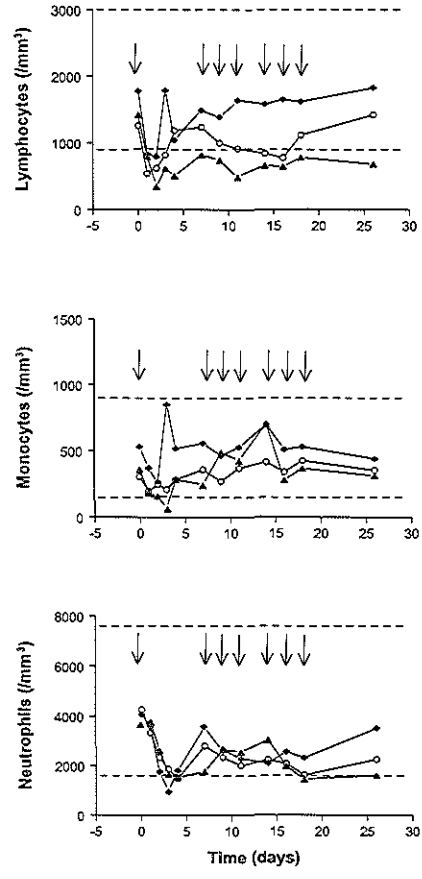

Figure 3. Leukocyte sub-populations after repeated administrations of IL-12. *Absolute numbers of lymphocytes, monocytes and neutrophils in the peripheral blood during 26-days following the first rHuIL-12 injection. Arrows depict rHuIL-12 administrations. Patients were treated with multiple doses of 0.1 µg/kg (◆: 3 patients), 0.5 µg/kg (O: 7 patients) or 1.0 µg/kg rHuIL-12 (▲: 2 patients). For each time point, the median result of each dose group is shown. Dotted lines indicate the lower and upper limits of the normal range.*

Table 2. Leukocyte subsets after the first subcutaneous administration of rHuIL-12

	0.1 μg/kg (n=3)				*0.5 μg/kg (n=15)*				*1.0 μg/kg (n=4)*			
	Baseline$^{\Psi}$ cell count		Nadir$^{\Psi}$		Baseline$^{\Psi}$ cell count		Nadir$^{\Psi}$		Baseline$^{\Psi}$ cell count		Nadir$^{\Psi}$	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
Lymphocytes	1.8	1.5 – 1.9	0.8**	0.5 – 0.8	1.3	0.5 – 2.9	0.5**	0.1 – 1.1	1.4	1.0 – 2.3	0.3*	0.2 – 0.5
Neutrophils	4.0	3.5 – 4.1	0.9	-	4.2	2.7 – 6.8	1.5**	0.7 – 4.7	3.7	2.9 – 5.3	1.4*	1.1 – 1.7
Monocytes	0.53	0.46 – 0.66	0.21	0.17 – 0.38	0.30	0.17– 0.58	0.15**	0.03– 0.43	0.35	0.29- 0.5	0.11*	0.02-0.16

* = $p < 0.05$; ** = $p<0.001$; nadirs compared to paired baseline counts.

Ψ Absolute numbers shown are x 10^9 / L

However, at the 0.5 and 1.0 μg/kg dose levels, neutrophils were still below baseline in 18 of 19 patients at 7 days post injection, whilst monocytes and lymphocytes were still below baseline levels in 10 out of 19 patients at that time (data not shown).

Repeated injections of rHuIL-12 had only minor effects on the numbers of circulating leucocyte subsets as compared to the first injection.

The subsequent injections at the 0.1 μg/kg dose level had no significant effects. At the 0.5 and 1.0 μg/kg dose levels, neutrophil counts were still below baseline levels, yet within normal ranges at 48 h after the 6th rHuIL-12 injection. However, at the highest dose levels, i.e. 1.0 and 1.25 μg/kg, a marked lymphopenia persisted during the entire period of follow-up (data not shown).

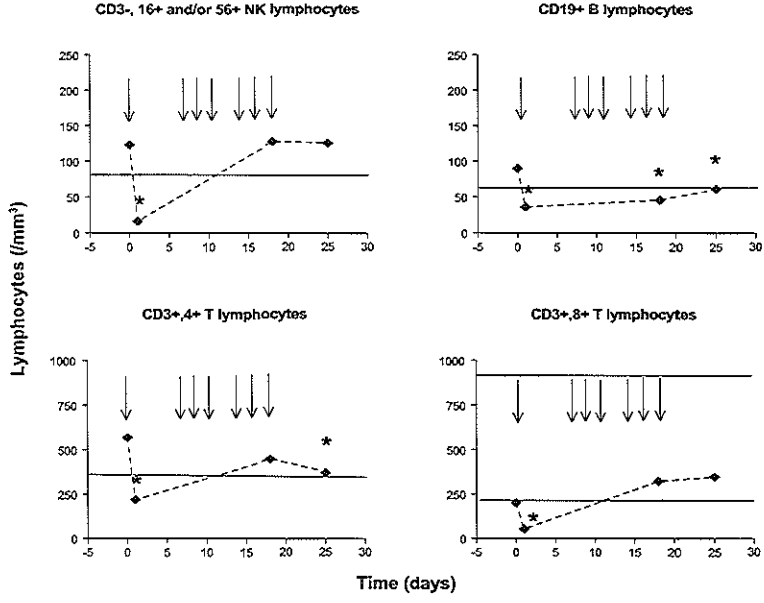

Figure 4. Lymphocyte sub-populations after repeated administrations of IL-12. *Absolute numbers of natural killer cells (CD3-,16+ and/or 56+), B-lymphocytes (CD19+), T-helper cells (CD3+,4+) and cytotoxic T-cells (CD3+,8+) during 26-days following the first rHuIL-12 injection. Arrows depict rHuIL-12 administrations. Patients either received a first injection of 0.5 μg/kg and subsequent injections either at the same dose level (n=4) or higher dose level (1.0 μg/kg (n=3); 1.25 μg/kg (n=2)). For each time point, the median result is shown. Dotted lines indicate the lower and (for CD3+, 8+ T cells) upper limits of the normal range. Significant differences between base-line cell counts and cell counts after IL-12 injection are indicated by an asterix (p< 0.05, paired samples).*

Lymphocyte subsets

Figure 4 shows the absolute numbers of NK lymphocytes (CD3-, 16+ and/or 56+), B lymphocytes (CD19+), T-helper cells (CD3+, 4+) and cytotoxic T cells (CD3+, 8+) before and after treatment with rHuIL-12.

Prior to treatment, all lymphocyte subsets were in the normal range. The first administration of 0.5 μg/kg rHuIL-12 induced significant reductions of all lymphocyte subset counts to levels below their normal ranges.

At 24 h after rHuIL-12 injection, NK cells had decreased from a median of 123 (range 38-314) to 16 (3-42) cells per mm^3 B cells from a median of 90 (range 21-240) to 36 (3-178) cells per mm^3 T-helper cells from a median of 569 (range 66-1262) to 219 (22-637) cells per mm3 and cytotoxic T cells from a median of 193 (range 240-854) to 59 (8-261) cells per mm^3 Immediately before and 7 days after the last injection of rHuIL-12, NK and cytotoxic T cells had returned to their baseline levels, whereas T-helper cell and, in particular, B-cell counts were still below these levels.

Discussion

Clinical side effects and IFN-γ induction decrease in the course of repeated IL-12 administrations to humans [Portielje et al, 1999; Leonard et al, 1997; Robertson et al, 1999; Bajetta et al, 1998; Rakhit et al, 1999; Haicheur et al, 2000; Gollob et al, 2000]. Here, we show that other immunological effects of IL-12 are also down-regulated in the course of systemic IL-12 treatment, such as declining TNF-α, IL-8 and IL-6 responses and diminishing effects on leucocyte subsets, and that maintenance of detectable concentrations of IFN-γ, as well as TNF-α, IL-8 and IL-6, can not be achieved by dose escalation of IL-12. A previous phase I study has shown an association between antitumor response and the maintenance of IFN-γ concentrations after repeated injections [Gollob et al, 2000]. These combined results indicate that a generalized down-regulation of immunological effects, possibly including anti-tumor effects, occurs upon repeated administrations of IL-12.

We showed that the plasma levels of the inhibitory cytokine IL-10 remained elevated or further increased upon repeated IL-12 injections. This observation has also been made in other clinical trials [Bajetta et al, 1998; Haicheur et al, 2000; Zeuzem et al, 2000]. An important role has been proposed for IL-10 in the down-regulation of IFN-γ production, as anti-IL-10 antibodies neutralized the

down-regulation of side effects upon repeated IL-12 administrations to mice, and IL-10 inhibited IL-12-mediated production of IFN-γ by human lymphoid cells [Sacco et al, 1997; Bajetta et al, 1998; Haicheur et al, 2000; D'Andrea et al, 1993; Pai et al, 1998]. Based on our results we suggest that IL-10 down-regulates the IL-12-mediated production of other cytokines as well. Indeed, *in vitro* studies have shown that IL-10 inhibits TNF-α production by lymphocytes [D'Andrea et al, 1993], IL-6 and IL-8 production by monocytes and macrophages [Fiorentino et al, 1991; Hodge-Dufour et al, 1998], and IL-8 production by neutrophils [Wang et al, 1994]. In various pathological states, endogenously produced IL-10 has an important function in the abrogation of ongoing inflammatory responses by inhibiting the effects mediated by endogenously produced IL-12 [Meyaard et al, 1996; Van der Poll et al, 1997; De Waal Malefyt et al, 1991; Jansen et al, 1996]. Hence, IL-12-induced IL-10 production appears to be a protective feedback mechanism. The induction of IL-10 seems to be independent of IFN-γ as neutralizing anti-IFN-γ antibodies had no effect on IL-12 induced IL-10 synthesis in vitro [Windhagen et al, 1996]. On the other hand, TNF-α possibly plays a role in the increased IL-10 production after IL-12 administration, as it induced high levels of IL-10 mRNA expression and release of IL-10 by human peripheral blood monocytes [Wanidworanun et al, 1993]. Moreover, TNF-α was shown to inhibit IFN-γ-mediated effects on human macrophages, and the inhibition of these effects by anti-IL-10 antibodies confirmed the intermediate role of IL-10 [Hodge-Dufour et al, 1998].

IL-18 is a pleiotropic cytokine that initially was discovered as IFN-γ inducing factor derived from liver cells [Okamura et al, 1995]. IL-18 synergizes with IL-12 to stimulate IFN-γ production by T cells [Mikallef et al, 1996]. In a phase I study of IL-12 to cancer patients, induction of IL-18 and IFN-γ were not correlated; upon repeated IL-12 administrations, IL-18 induction was sustained, whilst IFN-γ induction was downregulated [Gollob et al, 2000]. In addition, IL-18 plasma levels peaked later than those of IFN-γ [Gollob et al, 2000]. Extrapolation of these observations to our results would therefore suggest that the kinetics of IFN-γ plasma levels in our study are independent of IL-18.

Finally, the downregulation of IL-12-mediated effects upon repeated IL-12 administration may be due to the specific downregulation of its own signaling [Wang et al, 2001]. *In vitro,* prolonged stimulation of T cells by IL-12 results in depletion of the signal transducer and activator of transcription 4 (STAT4) protein. Downregulation of STAT4, a critical IL-12 signaling component, resulted in decreased IFN-γ production [Wang et al, 2001]. In line with these findings, we observed a generalized reduction of biological effects *in vivo* upon repeated

adminstration of IL-12 at doses approximating the maximum tolerated dose [Portielje et al, 1999].

We have shown that the administration of IL-12 to humans resulted in the release of TNF-α, IL-8 and IL-6, in addition to that of IFN-γ and IL-10. The dose-dependent induction of multiple cytokines after the first injection of IL-12 is probably a combination of direct and indirect IL-12 mediated effects. Activated T and NK cells and, as shown recently, neutrophils, eosinophils and dendritic cells [Desai et al, 1992; Collison et al, 1998; Nutku et al, 2001, Nagayama et al, 2000] express IL-12 receptors and IL-12 potentially mediates the production of secondary cytokines by binding to these cell populations. With respect to TNF-α, our results are in accordance with previous reports of elevated plasma TNF-α concentrations and increased TNF-α mRNA expression in peripheral blood mononuclear cells of patients with advanced cancer treated with IL-12 [Ohno et al, 2000; Haicheur et al, 2000]. *In vitro*, IL-12 stimulates the production of TNF-α by activated T cells and NK cells [Naume et al, 1992; Aste-Amezaga et al, 1994]. As monoclonal antibodies to TNF-α abrogate IL-12 mediated IFN-γ production in response to various stimuli *in-vitro* [D'Andrea et al, 1993; Tripp et al, 1993], TNF-α may be an essential co-stimulator of IFN-γ production and therefore an important intermediate in the anti-tumor effects of IL-12.

One study has addressed the issue of IL-8 concentrations after IL-12 in humans and described inconsistent patterns of stimulation [Bajetta et al, 1998]. In contrast, we observed induction of IL-8 in the vast majority of patients. TNF-α is a physiological stimulant of IL-8 production in humans [Van der Poll et al, 1992], and therefore, the induction of IL-8 may be secondary to TNF-α induction by rHuIL-12 in our study. Cells from the monocyte and macrophage compartment, endothelial cells and neutrophils are among the cells that can be stimulated by TNF-α to produce IL-8 [Baggiolini et al, 2000; Ethuin et al, 2001]. Alternatively, IL-8 induction may be a direct effect of rHuIL-12, as this cytokine was shown to induce the production of IL-8 from purified NK-cells [Naume et al, 1993b].

We also demonstrated that IL-6 peaks within 12 hours after s.c. IL-12 administration. Previous studies that addressed IL-6 reported large variation of plasma concentrations among patients and lack of IL-12 dose dependency [Bajetta et al, 1998; Ohno et al, 2000]. IL-6 peak levels may have been missed in these studies as the the first blood samples were not taken until 24 hours after IL-12 administration. Although TNF-α can stimulate IL-6 induction in humans [Van Snick, 1990], it probably had no major impact on IL-6 production in our patients, since IL-6 already reached peak levels 36 hours before TNF-α. Rather,

stimulation of IL-6 production may be directly mediated by IL-12, as binding of IL-12 to its receptors on dendritic cells has been shown to stimulate the production of IFN-γ, TNF-α and IL-6 at the transcription level [Nagayama et al, 2000]. IL-6 may also be considered as a natural feedback inhibitor of IL-12 production, as IL-6 inhibits both T-cell dependent and independent induction of IL-12 production in humans [Takenaka et al, 1997].

The transient reduction of lymphocyte, monocyte and neutrophil counts following the first IL-12 injection confirms previous observations [Robertson et al, 1999; Bajetta et al, 1998; Atkins et al, 1997]. We consider this pattern to reflect the transient redistribution of leukocytes, which adhere to endothelium and migrate into the tissues. Indeed, postmortem examination of animals treated with IL-12 showed massive infiltrates of leucocytes in lymph nodes, lungs, liver and spleen [Car et al, 1999]. The accumulation of NK and T cells in tumor nodules after IL-12 administration seems relevant to explain the putative anti-tumor effects of IL-12 [Mortarini et al, 2000; Allavena et al, 1994; Fogler et al, 1998]. Although in-vitro studies have demonstrated that IL-12 directly promotes interactions between endothelial cells and T cells, NK cells and neutrophils [Allavena et al, 1994; Colantonio et al, 1999], secondary cytokines such as IFN-γ, TNF-α and IL-8 may also contribute to the redistribution of leukocytes, as these cytokines enhance the expression of a wide range of molecules that regulate leukocyte adhesion and migration [Male, 1995]. In addition, chemokines induced by IFN-γ, such as IFN-γ-inducible protein 10 (IP-10) and monokine induced by IFN-γ (MIG) have potent chemotactic effects on T cells and may have contributed to their distribution [Haicheur et al, 2000]. As with IFN-γ, induction of these chemokines declines upon repeated IL-12 administrations [Haicheur et al, 2000].

In patients with advanced melanoma treated with s.c. IL-12, serum levels of the soluble endothelial adhesion molecules E-selectin, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 were transiently increased after IL-12 administration [Mortarini et al, 2000]. In parallel, the expression of their respective ligands, i.e., cutaneous lymphocyte antigen (CLA), very late antigen (VLA)-4 and lymphocyte function associated antigen (LFA)-1, were increased on circulating T cells. Thus, the enhanced expression of both receptors and ligands on endothelial cells and T lymphocytes may have promoted the marked infiltration of the melanoma lesions by tumor-specific CD8+ T cells observed in this study [Mortarini et al, 2000].

The development of IL-12 as a cancer therapeutic has followed the classical approach, starting with phase I studies, followed by efficacy studies with the

maximum tolerated dose that was previously defined. Our results indicate that the disappointing anti-tumor effects observed in phase II studies are possibly due to a generalized reduction of biological effects that occurs when IL-12 is repeatedly administered at doses and in schedules that approximate the maximum tolerated dose.

We conclude that the systemic administration of IL-12 results in direct and indirect induction of multiple cytokines. Upon repeated IL-12 administration, levels of pro-inflammatory cytokines diminish as well as effects on peripheral blood leucocyte subsets, while IL-10 production increases and likely contributes to the down-regulation. Dose escalation of IL-12, within tolerable margins, does not prevent the down-regulation of immunological effects. At present, IL-12 is being studied as an adjuvant for cancer vaccination. The present study indicates that the effects of IL-12 are downregulated when it is administered at dose levels near the maximum tolerated dose. Therefore, further investigations are required to define the dose and schedule of IL-12 with optimal immunological effects in the vaccination setting.

Chapter 4

Interleukin 12 induces activation of fibrinolysis and coagulation in humans

Adapted from:

Interleukin 12 induces activation of fibrinolysis and coagulation in humans

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British Journal of Haematology 2001; 112: 499-505

Abstract

Interleukin 12 has potential efficacy in malignant, infectious and allergic diseases. Its side-effects include activation of coagulation and fibrinolysis as documented in chimpanzees. We assessed the coagulative and fibrinolytic response in 18 patients with renal cell carcinoma after subcutaneous injection of 0.5 μg/kg recombinant human IL-12.

IL-12 induced a fibrinolytic response in 17 patients (94%): Plasmin-α2-antiplasmin complexes (PAPc) increased from 11.8 ± 6.6 nmol/l (mean ± SD) to a maximum of 18.8 ± 7.4 nmol/l at 72 hours. Baseline levels of tissue plasminogen activator (tPA) and plasminogen-activator inhibitor-I (PAI) were elevated in eight and 14 patients, respectively. tPA Increased from 12.6 ± 5.2 ng/ml to a maximum of 19.0 ± 6.7 ng/ml at 72 hours. PAI decreased from 111 ± 69 ng/ml to a minimum of 65 ± 53 ng/ml at 8 hours, thereafter remaining below baseline. Elevation of PAPc correlated with elevation of tPA and reduction of PAI.
A coagulative response occurred in nine patients (50%): Thrombin-anti-thrombin III complexes increased from 29 ± 53 ng/ml to a maximum of 460 ± 322 ng/ml at 12 hours. Patients with and without a coagulative response had similar levels of rHuIL-12, IFN-γ or TNF-α.

Conclusion: IL-12 can activate both fibrinolysis and coagulation in a significant proportion of patients with cancer. The time-frame and sequence of these activation processes differ from those known for other cytokines.

Introduction

Interleukin 12 (IL-12) is a cytokine with an important function in the regulation of the cell-mediated immune response. It stimulates the cellular immunity by promoting T-helper 1 responses while inhibiting T-helper 2 development and function and, therefore, may be used to restore dysbalances between Th-1 and Th-2 cells as may occur in malignant, infectious or allergic diseases [Manetti et al, 1993; Wu et al, 1993; Scott et al, 1993]. Additionally, IL-12 can inhibit tumor-associated angiogenesis [Voest et al, 1995; Sgadari et al, 1996; Coughlin et al, 1998]. In animal models it was demonstrated that IL-12 affects coagulation and fibrinolysis [Ozmen et al, 1994; Lauw et al, 1999]. In mice, IL-12 is an important mediator of the generalized Shwartzman reaction, a systemic inflammation with widespread thrombosis and disseminated intravascular coagulation [Ozmen et al, 1994]. Several cases of disseminated intravascular coagulation were observed after the administration of 5 μg/kg IL-12 to chimpanzees with viral hepatitis (personal communication T. Man, Roche). In healthy chimpanzees, the intravenous administration of 1 μg/kg IL-12 induced activation of coagulation and fibrinolysis [Lauw et al, 1999]. Whether the administration of IL-12 in humans is also associated with changes in coagulation or fibrinolysis is unknown.

An important immune-regulatory effect of IL-12 is the production of IFN-γ by T-lymphocytes and natural killer cells and many of the biological effects of IL-12 therapy in humans are attributed to the induction of this cytokine [Kobayashi et al, 1989; Chan et al, 1991]. IFN-γ, in turn, stimulates monocytes and macrophages to secrete tumor necrosis factor-alfa (TNF-α) that has been shown to activate the common pathway of coagulation in humans [Van der Poll et al, 1990].

To evaluate the involvement of IL-12 on coagulation and fibrinolysis, markers of coagulation and fibrinolysis were studied in patients with advanced renal cell carcinoma (RCC), treated in a phase I study with subcutaneous recombinant human IL-12 (rHuIL-12) at the maximum tolerated dose. Additionally, the pharmacokinetics of rHuIL-12 were studied and levels of IFN-γ and TNF-α were measured.

Materials and Methods

Patients

Patients had advanced renal cell cancer and participated in an open label non-randomized phase I dose escalation trial carried out in two European cancer centers to evaluate the safety and tolerability of an initial single injection of rHuIL-12 as well as the safety and tolerability of repeated s.c. injections. Toxicity analysis of the phase I study had indicated that 0.5 μg/kg was the maximum tolerated dose of a first s.c. injection of rHuIL-12 [Portielje et al, 1999]. Parameters of coagulation and fibrinolysis were studied in 18 patients after the first subcutaneous injection of rHuIL-12 at a dose of 0.5 μg/kg. The group consisted of 12 males and 6 females with a mean age of 57 years (range 42-70). Patients had a World Health Organization (WHO) performance score of 0 to 1, a life expectancy ≥ 4 months, adequate hematological status and normal renal, hepatic, cardiovascular and pulmonary function. Results of routine coagulation assays (prothrombin time and activated partial thromboplastin time) were normal. All former therapies were ended at least 6 weeks prior to start of treatment with rHuIL-12. Patients did not use systemic corticosteroids or anticoagulant drugs. Patients with major concurrent systemic disease were excluded. Patients gave informed consent and the ethics committees of participating hospitals approved of the protocol. Recombinant human IL-12 (rHuIL-12, Ro 24-7472) was supplied by Hoffmann La Roche, Nutley, U.S.A, and administered by s.c. injection. All patients received the injection at 8.00 a.m. Acetaminophen was prescribed to alleviate fever, headache and myalgia. Metoclopramide was prescribed in case of nausea and vomiting. No other medications were given routinely.

Collection of blood

Blood samples for coagulation and cytokine assays and platelet counts were obtained directly before and 4, 8, 12, 24, 48 and 72 hours after the first rHuIL-12 administration (0.5 μg/kg subcutaneously). In 12 patients blood was also obtained after 96 and 168 hours. Blood was drawn through an indwelling intravenous infusion needle (Venflon, 16 gauge). Blood for coagulation assays was collected in 5 ml siliconized glass tubes containing 10 mM EDTA, 10 mM benzamidine and 100 μg of soybean trypsin inhibitor (type I-S, Sigma chemicals, St Louis, USA) (final concentrations) to prevent activation of the complement and contact coagulation system. Plasma was obtained by centrifugation of blood for 10 minutes at 1300 g. Plasma samples were stored at −70°C until tested.

Cytokines and platelet counts were determined in EDTA anticoagulated plasma. Blood samples for measurement of prothrombin time (PT) and activated partial thromboplastin time (APTT) were obtained before and 24 and 168 hours after rHuIL-12 injection and collected in siliconized glass tubes containing 0.105 M sodium-citrate.

Coagulation and fibrinolysis assays

Plasmin-α2-antiplasmin (PAP) complexes, reflecting the activation of plasminogen, were measured as parameter of fibrinolysis. PAP complexes were determined with a radio-immuno assay described elsewhere in detail [Levi et al, 1992]. Normal values are less than 7 nmol/l. Antigenic levels of tissue type plasminogen (tPA) were measured with a previously described sandwich ELISA [Zonneveld et al, 1987]. The results of tPA measurements were related to standard curves of recombinant tPA, with normal values for tPA below 11 ng/ml. Levels of plasminogen-activator inhibitor-I (PAI-I) were assessed with an ELISA, that had been modified from a sandwich type radio-immunoassays and has been described elsewhere in detail [Boer et al, 1991]. In short, monoclonal anti-PAI-1 antibody (mAb CLB-2C8) was used as the coating antibody and biotinylated polyclonal rabbit anti-PAI-1 antibodies as the conjugate. Results were related to a standard curve of human PAI-1 and normal values were between 30 and 60 ng/ml. Thrombin-antithrombin III (TAT) complexes were measured with an enzyme linked immunosorbent assay described elsewhere in detail [Boermeester et al, 1995]. Normal values in this assay were less than 4 ng/ml.
PT and APTT were measured with a commercially available kit (PT-fibrinogen recombinant and APTT-SP (liquid) kit, Instrumentation laboratory, Barcelona, Spain).

Cytokine assays

Serum concentrations of bio-active IL-12 are measured by a method of antibody capture followed by a cell proliferation assay with a lower limit of detection of 50 pg/ml [Motzer et al, 1998]. IFN-γ and TNF-α concentrations were determined with commercially available enzyme amplified sensitivity immunoassays (Medgenic EASIA, Biosource Europe, Fleurus, Belgium). Normal values of IFN-γ are less than 0.2 IU/ml and normal values of TNF-α are less than 20 pg/ml. The lower limit of detection of the IFN-γ assay is 0.03 IU/ml and the lower limit of detection of the TNF-α assay is 3 pg/ml.

Pharmacologic data analyses

Individual plasma concentration-time data of cytokines were analysed by non-compartmental methods using the Siphar software package (version 4.0; SIMED, Créteil, France). Pharmacodynamic analysis of coagulation and fibrinolysis modulation by rHuIL-12 was also performed using the Siphar software. Total area under the effect curve ($AUEC_{total}$) for PAP complexes, tPA, PAI and TAT complexes were calculated for each patient using the trapezoidal rule. The $AUEC_{net}$, the area under the effect curve above baseline values, was calculated by [$AUEC_{total}$ minus $AUEC_{net}$]. The baseline levels of PAP complexes, tPA, PAI and TAT complexes were obtained from measured predose levels, assuming that baseline values would have been maintained for the duration of the study in the absence of rHuIL-12 administration.

Relationships between the various $AUEC_{net}$ values and between concentration-time profiles of PAP complexes, tPA, PAI and TAT complexes were evaluated by multiple regression analysis. To test parameter differences for statistical significance, a two-tailed unpaired Student's t-test was performed. Probability values of less than 0.05 were regarded as statistically significant. All calculations were performed using Number Cruncher Statistical System (NCSS, version 5.X; Dr. Jerry Hintze, Kayesville, UT, USA.

Results

Side effects

Common rHuIL-12 related side effects and laboratory abnormalities were fever, other flu-like symptoms, nausea, vomiting, fatigue, anemia, leucopenia, and hyponatremia. Liver enzymes increased in 7 patients, the maximum increase being 5 times the normal level. A more detailed decription of the clinical side effects has been given in our report of the phase I study [Portielje et al, 1999]. No hemorrhagic or thrombotic complications occurred within the first week after administration of the first dose of rHuIL-12.

Plasmin-α2-antiplasmin (PAP) complexes

Fourteen patients had elevated PAP complexes before rHuIL-12 administration with values ranging from 8.7 to 31 nmol/l (Table 1). After rHuIL-12, PAP complexes increased in 17 patients with individual peak concentrations reached between 12 and 168 hours (median 72).

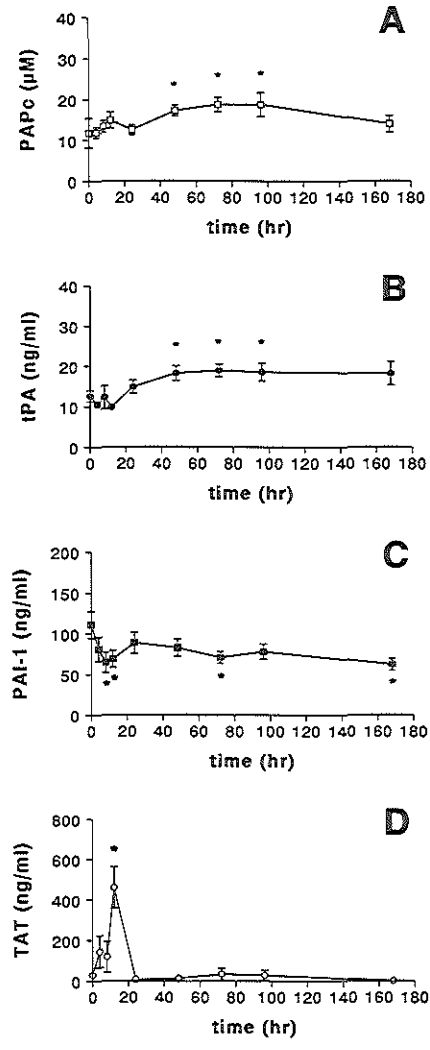

Figure 1. The effect of IL-12 on the fibrinolytic and coagulation systems. *Mean ± standard error of the mean (SEM) plasma concentrations of plasmin-α2-antiplasmin complexes (A), tissue plasminogen activator (B), plasminogen activator inhibitor-1 (C) and thrombin-anti-thrombin III complexes (D) during a period of 168 hours after a single subcutaneous injection of 0.5 µg/kg IL-12 in 18 patients with advanced renal cell carcinoma. Significant differences between base-line concentrations and concentrations after IL-12 injection are indicated by an asterix ($p < 0.05$).*

The mean concentration of PAP complexes increased from a baseline level of 11.8 ± 6.6 nmol/l to a maximum of 18.8 ± 7.4 nmol/l (p=0.005). Levels slowly declined without returning to baseline within the 7 days period of monitoring. Figure 1A shows the mean concentration of PAP complexes after rHuIL-12 injection. PAP levels remained constant in one patient.

Tissue plasminogen activator (tPA) and plasminogen-activator inhibitor-I (PAI)

At baseline, tPA levels were increased in 8 patients (range 12 – 24 ng/ml) and PAI levels were elevated in 14 patients (range 64 – 278 ng/ml) (Table 1). Seven out of 8 patients with baseline elevated tPA levels also had increased baseline PAI levels (Table 1).

The administration of rHuIL-12 induced an increase of circulating tPA in all patients, with individual peak concentrations reached between 48 and 168 hours (median 72). The mean concentration of tPA increased from a baseline level of 12.6 ± 5.2 ng/ml to a maximum of 19.0 ± 6.7 ng/ml (p=0.005). Thereafter, levels slowly decreased but had not reached baseline at 168 hours. At 168 hours, with samples available from 12 patients, mean tPA level was 18.3 ± 10.2 ng/ml versus a paired mean level of 11.7 ± 4.0 ng/ml (p=0.059) before therapy. Notably, in 6 out of 8 patients with increased baseline tPA levels, tPA initially decreased after rHuIL-12, from a mean baseline concentration of 17.7 ± 3.8 ng/ml to a minimum 9.7 ± 3.8 ng/ml, but then started to rise at 4 to 12 hours (median 8).

Table 1. Number of patients with elevated baseline levels of coagulative and fibrinolytic parameters, IFNγ or TNFα and median values.

Parameter (normal value)*	*No. of patients (%)*	*Median value*	*Range*
TATc (<4 ng/ml)	4 (22)	56 ng/ml	30 – 189
PAPc (<7 nmol/l)	14 (78)	12 nmol/l	8.7 – 31
tPA (<11 ng/ml)	8 (44)	16 ng/ml	12 – 24
PAI (30 – 60 ng/ml)	14 (78)	117 ng/ml	64 – 278
IFNγ (<0.2 IU/ml)	8 (44)	1.0 IU/ml	0.4 – 3.6
TNFα (<20 pg/ml)	14 (78)	31 pg/ml	22 – 83

*Plasma concentration measured in healthy individuals.

In all 14 patiens with elevated PAI at baseline, levels decreased after rHuIL-12, with individual minimum concentrations reached after 4 to 168 hours (median 8). PAI levels did not change in the 4 patients with normal levels at baseline. The mean concentration of PAI decreased from a baseline level of 111 ± 69 ng/ml to a minimum of 65 ± 53 ng/ml (p=0.031). After the nadir, PAI levels slowly increased but remained below baseline during the whole period of monitoring. Figures 1B and C show the mean concentrations of tPA and PAI after rHuIL-12 injection.

Elevation of PAP complexes after rHuIL-12 injection correlated significantly with elevation of tPA and reduction of PAI (p=0.0042).

Thrombin-anti-thrombin III (TAT) complexes

TAT complexes were measured to assess thrombin generation induced by IL-12. RHuIL-12 induced a coagulative response in 9 patients (50%), with individual peak levels reached between 4 to 72 hours (median 12). The mean concentration of TAT complexes increased from a baseline level of 29 ± 53 ng/ml to a maximum of 460 ± 322 ng/ml (p=0.0001). Four patients had strongly elevated levels of TAT complexes before injection of rHuIL-12, with values between 30 and 189 ng/ml (Table 1). Within 4 hours after administration of rHuIL-12 to these patients, TAT complexes further increased, to reach maximal concentrations between 551 and 816 ng/ml. Of 14 patients with normal baseline levels of TAT complexes, 5 showed increasing levels upon rHuIL-12 administration with maximal concentrations of TAT complexes between 19 and 605 ng/ml. Levels of TAT complexes had normalized in all patients at 24 to 168 hours (median 48). Notably, although the patients had indwelling catheters, an artefactual increase of TAT complexes by these catheters can not explain the course of TAT levels, since such an artefactual increase is expected to occur immediately after insertion of the catheters. Figure 1D shows the mean concentration of TAT complexes after rHuIL-12 injection.
In all patients with detectable thrombin generation, peak levels of TAT complexes preceded peak levels of PAP complexes.

Routine clotting parameters

PT and APTT as well as platelets were measured in all patients. No significant changes were noted in PT or APTT values upon rHuIL-12 administration. In contrast, mean platelet count decreased after rHuIL-12 injection, from 295 ± 99 at baseline to a minimum of 203 ± 88 million per ml (p=0.008). Individual minimum counts were reached after 24 to 96 hours (median 72). Platelet counts

had returned to baseline levels at 168 hours. Two patients experienced grade 1 thrombocytopenia, with platelet counts between 75 and 100 million per ml.

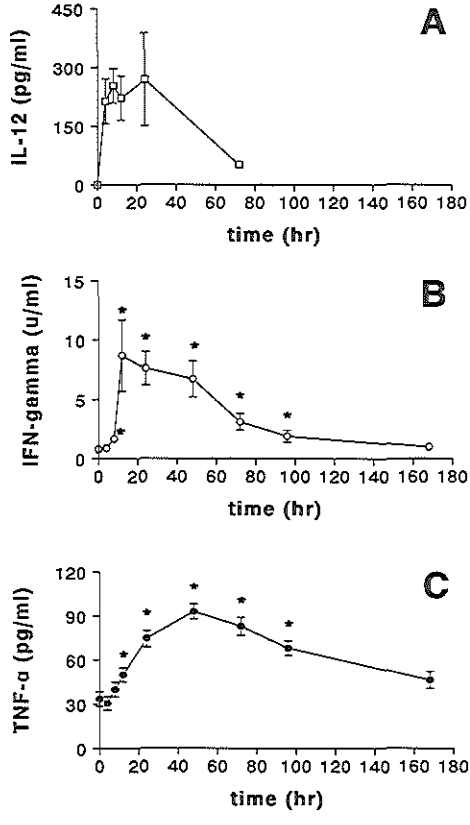

Figure 2. ***Plasma concentrations of IL-12, IFN-γ and TNF-α after IL-12 injection.***
Mean ± standard error of the mean (SEM) plasma concentrations of rHuIL-12 (A), IFNγ (B) and TNFα (C) during a period of 168 hours after a single subcutaneous injection of 0.5 μg/kg IL-12 in 18 patients with advanced renal cell carcinoma. Significant differences between base-line concentrations and concentrations after IL-12 injection are indicated by an asterix ($p < 0.05$).

Cytokines

Blood samples for IL-12 measurements were available from 17 patients. Before injection, serum IL-12 levels were below the detection limit in all patients. After injection, the mean concentration of IL-12 increased to 269 ± 291 pg/ml. Individual peak levels of rHuIL-12 were reached after 4 to 24 hours (median 12). Figure 2A shows mean levels of IL-12.

At base-line IFN-γ concentrations were increased in 8 patients with values between 0.4 and 3.6 IU/ml (table 1). IFN-γ increased in all patients, with individual maximum concentrations reached after 12 to 48 hours (median 24). The mean concentration increased from 0.8 ± 0.9 IU/ml to a maximum of 8.7 ± 12.4 IU/ml ($p= 0.025$). Thereafter IFN-γ slowly decreased to reach baseline levels at day 7 of observation. Base-line TNF-α concentrations were increased in 14 patients with values between 22 and 83 pg/ml (table 1).
In response to rHuIL-12, TNF-α levels increased in all patients with individual maximum levels reached after 24 to 72 hours (median 48). The mean plasma concentration increased from 33.5 ± 20.7 pg/ml to a maximum of 92.9 ± 21.8 pg/ml ($p< 0.00001$). Figures 2B and C show mean levels of IFN-γ and TNF-α after rHuIL-12 administration.

Serum concentrations of rHuIL-12 and pattern and height of IFN-γ and TNF-α induction were not different for patients with and without detectable thrombin response.

Discussion

IL-12 induced sustained activation of fibrinolysis in the majority and substantial thrombin generation in half of the patients. While the endothelium was stimulated to produce tPA, secretion of its natural inhibitor PAI-1 decreased. This opposite behaviour of tPA and PAI-1, as far as we know unique for IL12, probably explained the observed plasminogen activation. The fibrinolytic response continued at the time thrombin generation had already abated and, therefore, occurred independently, as has previously been observed in experimental endotoxemia [Van der Poll et al, 1991].

Coagulative and fibrinolytic responses to IL-12 have only been studied in chimpanzees. There, TAT complexes reached a plateau between 8 to 48 hours after intravenous administration and PAP complexes were maximal at the last sampling point at 48 hours, indicating a similar sustained response.

The mechanism by which IL-12 influences coagulation and fibrinolysis remains to be elucidated. Although many biological effects can be attributed to the induction of IFN-γ, this seems not a key mediator here. Not only were IFN-γ levels the same for patients with and without thrombin generation, the peak of the coagulative response preceded maximal IFN-γ levels by 12 hours. In in-vitro models, IFN-γ inhibited PAI-1 production by endothelial cells and suppressed PAI-1 mRNA expression [Gallacchio et al, 1996; Siren et al, 1994]. The literature yields conflicting reports on the in-vivo effects of IFN-γ: In dermatitis patients, IFN-γ inhibited fibrinolysis by suppressing the release of tPA and stimulating PAI. Concomitantly TAT complexes increased [Musial et al, 1998]. In patients with severe injuries, with baseline elevation of markers of both coagulation and fibrinolysis, IFN-γ did not further affect these systems [Dries et al, 1998]. Neither seems TNF-α the mediator of the observed effects. The height of TNF-α levels and the coagulative response were not correlated, and TNF-α peaked when thrombin response had completely extinguished. With regard to fibrinolysis, TNF-α administration was associated with a rapid early activation, unlike the response we observed [Van der Poll et al, 1991]. Also, in chimpanzees, induction of coagulation and fibrinolysis after IL-12 occurred without detectable TNF-α induction [Lauw et al, 1999]. Furthermore, in experimental endotoxemia in humans, thrombin generation was not affected by anti-TNF [Van der Poll et al, 1994a]. Apparently TNF-α does not always induce a coagulative response in vivo [Van der Poll et al, 1990].

Compatible with the observation that patients with malignancies frequently have activation of systemic coagulation and fibrinolysis [Zacharski et al, 1992], the majority of our patients had elevated baseline levels of the markers of fibrinolysis and coagulation. Moreover, alterations in the plasminogen activator-plasmin proteolytic systems appear to be essential events in tumor progression [Mignatti and Rifkin, 1993]. In a study with IL-6, patients with metastatic RCC had comparable elevated baseline levels of PAP complexes, PAI and tPA [Stouthard et al, 1996]. However, baseline levels of TAT complexes were considerably lower. Whether this difference reflects different tumor load is unclear.

IL-12 induced fibrinolytic and coagulative responses that are completely different from those induced by other cytokines. As opposed to other cytokines, thrombin generation preceded maximal fibrinolysis [Van der Poll et al, 1990 and 1991; Baars et al, 1992; Levi et al, 1993 and 1994]. Additionally, the coagulative and fibrinolytic responses were delayed and the duration of the fibrinolytic response was exceptionally long. For example, interleukin-2 and TNF-α caused

activation of coagulation and fibrinolysis within 6 hours after administration [Baars et al, 1992; Fleischmann et al, 1991; Van der Poll et al, 1990; Boermeester et al, 1995]. The subcutaneous route of administration may in part explain the delayed effects of IL-12, but intravenous administration also induced a prolonged activation of fibrinolysis [Lauw et al, 1999]. This protracted effect of IL-12 may be related to its relatively long half-life. While cytokines characteristically have a plasma half-life of less than 30 minutes, IL-12 has a half-life of more than 5 hours [Atkins et al, 1997; Portielje et al, 1999].

The clinical relevance of the observed effects of IL-12 on fibrinolysis and coagulation cannot be distilled from our data. No hemorrhagic or thrombotic complications occurred in our study. However, in previous studies several bleeding episodes were mentioned. When 0.5 μg/kg of intravenous IL-12 was administered on 5 consecutive days as part of a phase II study, 2 patients had gastrointestinal bleeding that complicated ulceration of the large intestine and died [Leonard et al, 1997]. In the preceding phase I study, 2 out of 12 patients treated with the same dose experienced severe gastrointestinal hemorrhage [Atkins et al, 1997]. An additional gastrointestinal bleeding episode was observed in a study with subcutaneous IL-12 [Motzer et al, 1998]. No details were provided concerning coagulation tests or platelet counts. Although inflammation and ulceration of the lining of the gastrointestinal tract are considered to be IL-12 related side effects, it can not be excluded that coexisting coagulation abnormalities prompted hemorrhage from the damaged mucosa.
We can only speculate whether stimulation of fibrinolysis contributes to the anti-tumor effect of IL-12. IL-12 is supposed to have an inhibitory effect on tumor-angiogenesis, though the underlying mechanism is yet unknown [Voest et al, 1995; Sgadari et al, 1996] Our results indicate that IL-12 inhibits PAI-1 production. Tumor-angiogenesis has recently been shown to be PAI-1 dependent and the increased PAI-1 levels in cancer patients appear to be causally contributing to tumor invasion [Bajou et al, 1998]. Further studies should reveal whether the observed fibrinolytic responses may result in inhibition of tumor angiogenesis.

In conclusion, we have shown that administration of IL-12 can induce sustained activation of the fibrinolytic system and a substantial thrombin generation. The timing and duration of these processes differ from those observed with other cytokines. Further investigations are needed to elucidate the mechanism behind these changes and the relation to anti-tumor and side effects.

Chapter 5

Subcutaneous injection of IL-12 induces systemic inflammatory responses in humans: implications for the use of IL-12 as vaccine adjuvant

Subcutaneous injection of interleukin 12 induces systemic inflammatory responses in humans: implications for the use of IL-12 as vaccine adjvant

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Abstract

IL-12 is a cytokine with important regulatory functions bridging innate and adaptive immunity. IL-12 has been proposed as an immune adjuvant for vaccination therapy of infectious diseases and malignancies. The inflammatory signals mediated by IL-12 can play an important role in the adjuvant effect, but at the same time may constitute a risk for side effects.

In the setting of a phase I study, we studied the effect of different doses of s.c. administered recombinant human IL-12 in 26 patients with advanced renal cell carcinoma, and now demonstrate that IL-12 induces dose-dependent systemic activation of multiple inflammatory mediator systems in humans. Previously we have shown that the maximum tolerated dose of IL-12, i.e. 0.5 μg/kg, induced a significant coagulative response, here we demonstrate that this dose also induces degranulation of neutrophils: plasma levels of elastase (azurophilic granules) and lactoferrin (specific granules) increased from 48 ± 17 ng/ml (mean ± standard deviation) to 117 ± 136 ng/ml ($p < 0.05$) and from 124 ±51 ng/ml to 181 ±82 ng/ml ($p=0.01$), respectively, at 24 hours. Additionally, IL-12 injection mediated the release of lipid mediators: Plasma concentrations of secretory phospholipase A_2 ($sPLA_2$) increased from 17 ± 20 ng/ml to 97 ± 113 ng/ml ($p=0.003$). Systemic activation of inflammation and coagulation by IL-12 occurred in a dose dependent way. At 0.1 μg/kg of IL-12, systemic responses were minimal: Although mild activation of neutrophils was already detectable, activation of the coagulative response did not yet occur, and activation of fibrinolysis and formation of $sPLA_2$ were limited. Because s.c. injection is a common route of administration for immune adjuvants, we suggest that IL-12 as adjuvant should not be given at doses higher than 0.1 μg per kg in order to avoid severe systemic inflammatory responses.

Introduction

Interleukin 12 (IL-12) is a cytokine with important immuno-regulatory functions. IL-12 stimulates T-cells and NK-cells to produce IFN-γ and increases cytotoxic activity by NK-cells. Additionally IL-12 stimulates the helper activity of CD4 T-cells towards cellular immune responses and enhances antigen specific CD8+ T cell responses [Trinchieri, 1998]. As a stimulator of the cellular immune response, IL-12 has potential efficacy in malignant, infectious and allergic diseases. Studies of systemic cytokine therapy with IL-12 have been performed in different types of cancer, asthma and viral hepatitis [Carreno et al, 2000; Hurteau et al, 2001; Bryan et al, 2000; Portielje et al, 1999; Atkins et al, 1997; Motzer et al, 1998 and 2001; Leonard et al, 1997]. However, IL-12, administered either intravenously or subcutaneously, had disappointing efficacy but substantial toxicity. Recently, it was proposed that IL-12 may be an effective immune adjuvant for vaccination therapy of infectious diseases and malignancies [Gherardi et al, 2001; Rodolfo et al, 1999; Buchanan et al, 2001]. The immunological mechanisms underlying the adjuvant efficacy of IL-12 are not fully defined. Classical adjuvant substances are strong stimulators of local inflammation and the pro-inflammatory characteristics of IL-12 are thought to contribute to its adjuvant effects. Similar to other adjuvants, the subcutaneous route also appears appropriate for IL-12 for this purpose.

Polymorphonuclear neutrophils (PMN) are important effectors of the inflammatory response since they release toxic compounds such as proteases upon activation, that damage the microenvironment. We do not know whether or not IL-12 activates neutrophils in humans. Few preclinical studies have addressed the effects of IL-12 on PMN. Following administration of IL-12 to chimpanzees, degranulation of PMN was observed [Lauw et al, 1999]. In-vitro studies have revealed that PMN express IL-12 β1 receptors and that binding of IL-12 results in actin polymerization and a concentration dependent increase in reactive oxygen metabolites in PMN [Collison et al, 1998]. In addition to potential direct effects through IL-12 receptor engagement, IL-12 could also exert indirect effects on PMN through the induction of other pro-inflammatory cytokines such as TNF-α, IL-6 or IL-8. The degranulation of PMN in various physiological situations is appropriately reflected by levels of elastase-α1-antitrypsin complexes and lactoferrin in the peripheral blood [Van der Poll et al, 1994a 1994b; Nuijens et al, 1992; Suffredini et al, 1989]. Elastase is a proteinase released from azurophilic granules of neutrophils that rapidly forms complexes

with its natural inhibitor α1-antitrypsin, while lactoferrin is derived from the specific granules [Weiss, 1989].

The increased release of secretory phospholipase A_2 ($sPLA_2$) from endothelial and other cells is considered as another component of the inflammatory cascade [Pruzanski & Vadas, 1991]. PLA_2 is a lipolytic enzyme that releases fatty acids, often arachidonic acid, from membrane phospholipids for production of important lipid mediators such as tromboxane A_2, prostaglandins, leukotriens and platelet activating factor [Crowl et al, 1991]. PLA_2 is thought to promote phagocytosis of injured cells and tissue debris, thereby enhancing inflammation [Pruzanski & Vadas, 1991; Hack et al, 1997]. During inflammatory reactions plasma levels of $sPLA_2$ may markedly increase up to 100-fold over base-line.

At present there are no data regarding the potential of locally injected IL-12 to induce systemic inflammatory responses in humans. We performed a phase I study with IL-12 in patients with advanced renal cell cancer [Portielje et al, 1999]. In the present analysis, we address systemic inflammatory and coagulation responses in these patients. Our results show that IL-12 dose-dependently triggers these responses. These data may be useful for the design of studies in which IL-12 is used as an adjuvant.

Materials and Methods

Patients

We studied 26 patients with advanced renal cell cancer which participated in a phase I dose escalation trial carried out in the Rotterdam and Mainz cancer centers to evaluate the safety and tolerability of s.c. administered rHuIL-12. Toxicity analysis of the phase I study indicated that 0.5 μg/kg was the maximum tolerated dose of the first s.c. injection of rHuIL-12 [Portielje et al, 1999]. We studied markers of PMN degranulation in these patients after the first s.c. injection of rHuIL-12 at a dose of 0.1 μg/kg (n=3), 0.5 μg/kg (n=19) or 1.0 μg/kg (n=4). Prior to treatment, patients had a World Health Organization (WHO) performance score of 0 to 1, and adequate hematological, renal, hepatic, cardiovascular and pulmonary functions. All former therapies were terminated at least 6 weeks prior to start of treatment with rHuIL-12. The patients did not use systemic corticosteroids. Patients with concurrent systemic disease were excluded. Patients gave informed consent and the ethics committees approved of the protocol. Recombinant human IL-12 (rHuIL-12, Ro 24-7472) was supplied by Hoffmann La Roche (Nutley, NJ) and administered by s.c. injection. All injections

were given at 8.00 a.m. Acetaminophen was prescribed to alleviate fever, headache and myalgia. Metoclopramide was prescribed in case of nausea and vomiting. No other medications were given routinely.

Blood sampling and assays

Blood samples for elastase-α1-antitrypsin complexes, lactoferrin and $sPLA_2$ measurement were obtained directly before and 4, 8, 12, 24, 48 and 72 hours after the first rHuIL-12 administration. In 12 patients blood was also obtained after 96 and 168 hours. Blood was drawn through an indwelling intravenous infusion needle (Venflon, 16 gauge). Plasma was obtained by centrifugation of blood for 10 minutes at 1300g. Plasma samples were stored at –70°C until tested. All assays were done with EDTA anti-coagulated plasma. Plasma levels of elastase-α1-antitrypsin complexes and lactoferrin were assayed by RIA as described in detail previously [Nuijens et al, 1992]. Briefly, sepharose beads, to which polyclonal antibodies against human elastase or a mAb against human lactoferrin were coupled, were incubated with the samples to be tested. Elastase-α1-antitrypsin or lactoferrin bound to the beads was quantitated by incubation with ^{125}I-mAb against complexed α1-antitrypsin (RIA for elastase-α1-antitrypsin) or polyclonal ^{125}I-antilactoferrin (RIA for lactoferrin). Results were expressed as nanograms of elastase complexes or lactoferrin per ml. by reference to standard curves. The lower limit of detection of elastase-α1-antitrypsin complexes is 25 ng/ml; normal values are less than 100 ng/ml. The lower limit of detection of lactoferrin is 100 ng/ml; normal values are less than 400 ng/ml.

Secretory phospholipase A_2 ($sPLA_2$) concentrations in plasma were determined with an ELISA that was modified from that reported by Smith [Smith et al, 1992]. MAbs against human $sPLA_2$ type II (provided by Dr. F.B. Taylor Jr., Oklahoma Medical Research Foundation, Oklahoma City, OK, USA) were used as the coating and catching antibodies, respectively. Results are expressed by reference to a standard curve consisting of a dilution of culture supernatant of HepG2 cells stimulated with IL-6, in which the amount of $sPLA_2$ was assessed by comparison with recombinant human secretory-type PLA_2 ($sPLA_2$; courtesy of Prof. H.M. Verheij, Department of Enzymology and Protein Engineering, University of Utrecht, Utrecht, The Netherlands). The lower limit of detection of this assay is 0.2 ng/ml. Normal values are less than 5 ng/ml.

Serum concentrations of bio-active IL-12 were measured by a method of antibody capture followed by a cell proliferation assay with a lower limit of detection of 50 pg/ml [Motzer et al, 1998]. Samples for IL-12 determination were available from 25 patients [Portielje et al, 1999]. IFN-γ, TNF-α, IL-10, IL-8,

and IL-6 concentrations were determined with commercially available enzyme amplified sensitivity immunoassays (Medgenic EASIA, Biosource Europe, Fleurus, Belgium). The lower limits of detection of the assays were: IFN-γ: 0.03 IU/ml; TNF-α: 3 pg/ml; IL-10: 1 pg/ml; IL-8: 1 pg/ml; IL-6: 2 pg/ml and IL-4: 0.2 pg/ml. Cytokine concentrations were measured as part of previous [Portielje et al, 1999] and ongoing studies and results from patients treated at dose level 0.5 μg/kg rHuIL-12 are presented here to enable definition of the relationship between cytokine concentrations and inflammatory parameters. Blood samples for determination of absolute numbers of peripheral-blood polymorphonuclear neutrophils (PMN's) were available from 20 patients and collected directly before and 1, 2, 3, 4 and 7 days after the first administration of rHuIL-12. PMN counts were determined in EDTA anti-coagulated blood samples using a Technicon H1 automated cell counter (Technicon, Tarrytown, NY, USA).

Plasmin-α2-antiplasmin (PAP) complexes, were measured as parameter of fibrinolysis. PAP complexes were determined with a radio-immuno assay described elsewhere in detail [Levi et al, 1992]. Normal values are less than 7 nmol/l. Thrombin-antithrombin III (TAT) complexes were measured with an enzyme linked immunosorbent assay described elsewhere in detail [Boermeester et al, 1995]. Normal values in this assay were less than 4 ng/ml.

Pharmacologic data analyses

Individual plasma concentration-time data of cytokines were analysed by non-compartmental methods using the Siphar software package (version 4.0; Inna Phase, Philadelphia, PA, USA). Pharmacodynamic analysis of the modulation of leucocyte counts, PMN degranulation products and $sPLA_2$ induction by rHuIL-12 was also performed using the Siphar software. Total area under the effect curve ($AUEC_{total}$) for elastase-α1-antitrypsin complexes, lactoferrin, and $sPLA_2$ was calculated for each patient using the trapezoidal rule. The $AUEC_{net}$, the area under the effect curve above baseline values, was calculated by [$AUEC_{total}$ minus $AUEC_{net}$]. The baseline levels of elastase-α1-antitrypsin complexes, lactoferrin and $sPLA_2$ and the baseline leucocyte counts were obtained from measured pre-dose levels and counts, assuming that baseline values would have been maintained for the duration of the study in the absence of rHuIL-12 administration.

Relationships between the various $AUEC_{net}$ values and between concentration-time profiles of elastase-α1-antitrypsin complexes, lactoferrin and $sPLA_2$ were evaluated by multiple regression analysis. To test parameter differences for statistical significance in the paired samples, a two-tailed paired Student's t-test

was performed. To test parameter differences for statistical significance in the unpaired samples, a two-tailed unpaired Student's t-test was performed. Probability values of less than 0.05 were regarded as statistically significant. All calculations were performed using Number Cruncher Statistical System (NCSS, version 5.X; Dr. Jerry Hintze, Kayesville, UT, USA).

Results

Neutrophil numbers

RHuIL-12 induced depression of the number of peripheral blood PMN in all patients. After injection of 0.1 μg/kg rHuIL-12, PMN decreased from 4.0 x 10^9/l to 0.94 x 10^9/l (n=1). Figure 1 shows that after injection of 0.5 μg/kg rHuIL-12 (n=15), PMN decreased from 4.55 ± 1.32 x 10^9/l (mean ± standard deviation) to 1.74 ± 0.84 x 10^9/l ($p < 0.001$).

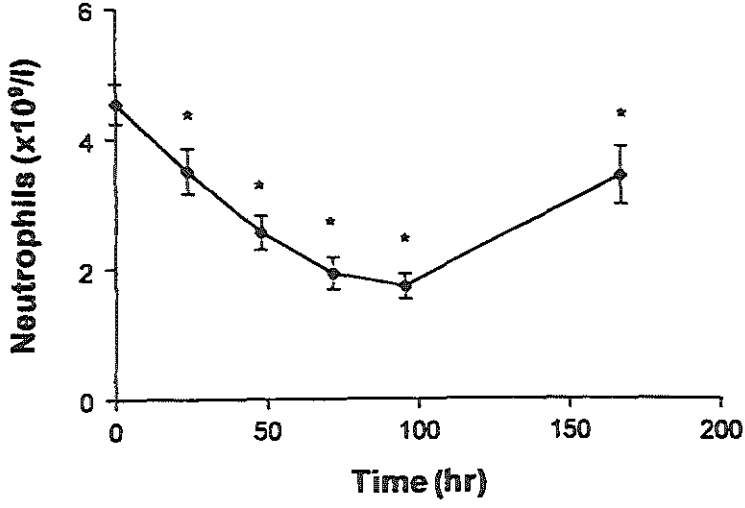

Figure 1. Neutrophil counts after subcutaneous IL-12.
Mean ± standard error of the mean (SEM) neutrophil counts during a period of 168 hours after a single subcutaneous injection of 0.5 μg/kg IL-12 in 14 patients with advanced renal cell carcinoma. Significant differences between base-line concentrations and concentrations after IL-12 injection are indicated by an asterix ($p<0.05$).

Finally, after injection of 1.0 μg/kg rHuIL-12 (n=4), PMN decreased from 3.9 ± 1.14 x 10^9/l to 1.45 ± 0.33 x 10^9/l. After 0.5 μg/kg rHuIL-12, PMN nadir occurred after a median of 3 days (range: 2-4) while after 1.0 μg/kg rHuIL-12, PMN nadir occurred after a median of 4 days (range: 4-7). At dose levels 0.5 and 1.0 μg/kg rHuIL-12, PMN remained significantly below baseline values ($p< 0.001$) during the whole 7- day observation period.

PMN degranulation

RHuIL-12 administration resulted in degranulation of PMN, reflected by (i) increased plasma levels of elastase-α1-antitrypsin complexes, which reflect the degranulation of azurophilic granules and (ii) increased plasma levels of lactoferrin, which reflect the degranulation of specific granules (figure 2).

Elastase-α1-antitrypsin complexes increased in all patients and increases were rHuIL-12-dose dependent (table 1). At doses of 0.1 and 0.5 μg/kg rHuIL-12, elastase-α1-antitrypsin complexes peaked after a median of 48 hours (range: 8-72), whereas at doses of 1.0 μg/kg rHuIL-12, maximum concentrations were reached after a median of 60 hours (range: 48-72). Plasma concentrations of lactoferrin increased in 23 out of 26 patients (89%). Individual maximum plasma concentrations of lactoferrin varied considerably among patients and patients with low baseline levels tended to have relatively low peak levels as well.

At doses of 0.1 and 0.5 μg/kg rHuIL-12, lactoferrin peaked after a median of 24 hours (range: 4-96), whereas at doses of 1.0 μg/kg rHuIL-12, maximum concentrations of lactoferrin were reached after a median of 60 hours (range: 48-72). Elevation of elastase-α1-antitrypsin complexes after rHuIL-12 injection correlated significantly with elevation of lactoferrin levels ($r^2 = 0.24$, $p=0.03$).

Plasma concentrations of $sPLA_2$

Levels of circulating $sPLA_2$ were measured as an indirect parameter for the formation of lipid mediators such as tromboxane A_2, prostaglandins, leukotriens and platelet activating factor.

RHuIL-12 induced an increase of circulating $sPLA_2$ in 25 of 26 patients and elevation was IL-12 dose dependent (table 1). Nine patients had elevated $sPLA_2$ concentrations before rHuIL-12 administration, with values ranging from 6.9 to 63 ng/ml. Patients with elevated baseline concentrations had significantly higher peak levels than patients with normal baseline levels (205 ± 149 (mean ± standard deviation) versus 31 ± 17, $p < 0.01$). Figure 3 shows the mean concentration of $sPLA_2$ complexes after 0.5 μg/kg rHuIL-12.

The median time to peak concentration was 48 hours after a dose of 0.1 and 0.5 µg/kg rHuIL-12, and 72 hours after a dose of 1.0 µg/kg. Individual peak concentrations were reached between 48 and 168 hours. Levels slowly declined to baseline within the 7 days of observation.

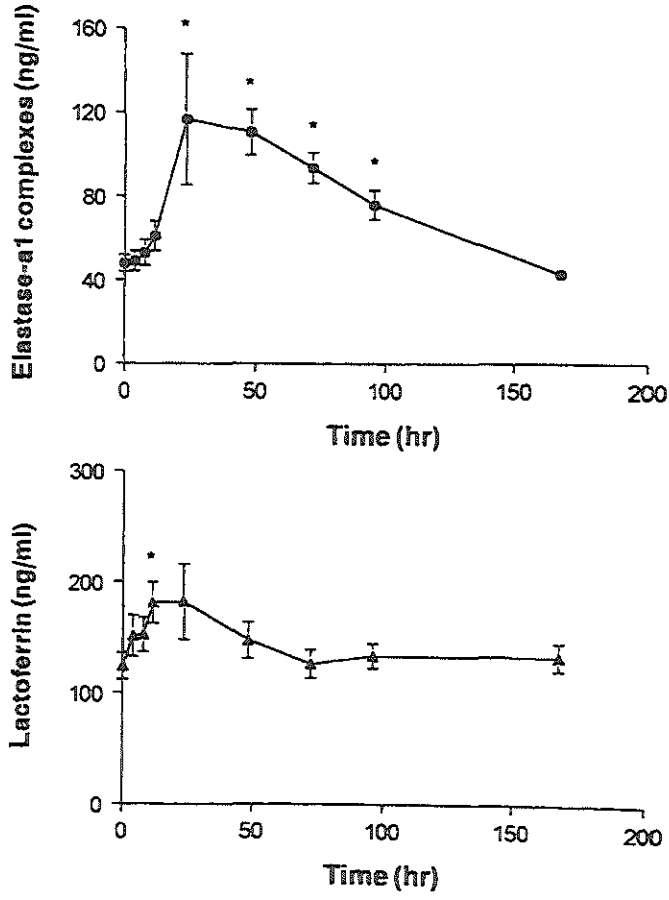

Figure 2. Neutrophil degranulation after subcutaneous IL-12.
Mean ± standard error of the mean (SEM) plasma concentrations of elastase-α1-antitrypsin complexes (A) and lactoferrin (B) during a period of 168 hours after a single subcutaneous injection of 0.5 µg/kg IL-12 in 19 patients with advanced renal cell carcinoma. Significant differences between base-line concentrations and concentrations after IL-12 injection are indicated by an asterix ($p<0.05$).

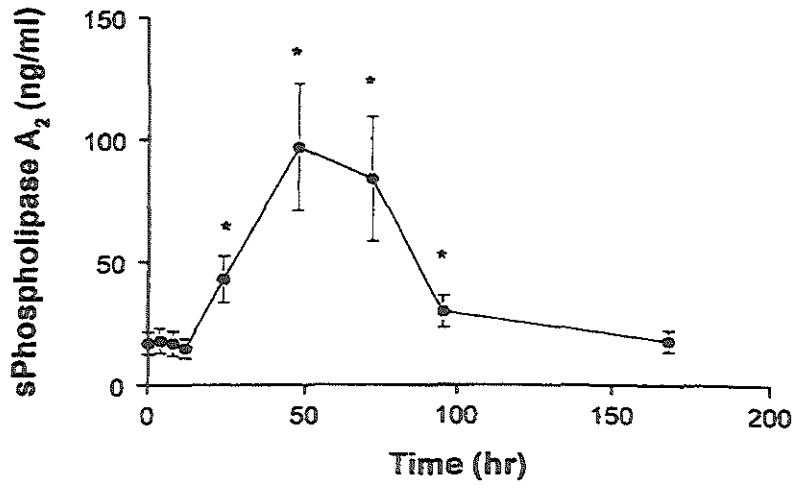

Figure 3. Plasma concentrations of secretory phospholipase A_2 ($sPLA_2$) after subcutaneous IL-12.
Mean ± standard error of the mean (SEM) plasma concentrations of $sPLA_2$ during a period of 168 hours after a single subcutaneous injection of 0.5 µg/kg IL-12 in 19 patients with advanced renal cell carcinoma. Significant differences between base-line concentrations and concentrations after IL-12 injection are indicated by an asterix ($p<0.05$).

Coagulation and fibrinolysis

Plasmin-α2-anti-plasmin (PAP) complexes were measured as a parameter for the activation of plasminogen. RhuIL-12 administration resulted in activation of fibrinolysis in all patients with IL-12 dose dependent elevation of plasma concentrations of PAP complexes (table 1). PAP complexes peaked after a median of 72 hours (range: 12-168). Thereafter, levels slowly declined, but did not reach baseline within the first week after rHuIL-12 administration.

Table 1. Circulating inflammatory parameters after subcutaneous administration of rHuIL-12

	Baseline (all)		0.1 µg/kg (n=3)		0.5 µg/kg (n=19)		1.0 µg/kg (n=4)	
	Mean	SD	Mean♣	SD	Mean♣	SD	Mean♣	SD
Elastase-α1-ATc (<100ng/ml)	46	16	87	35	117*	136	202*	79
Lactoferrin (<400ng/ml)	122	45	197	85	181*	82	248	112
$sPLA_2$ (<5ng/ml)	14	18	23	17	97*	113	144	99
PAPc (<7nmol/l)	10.5	6.2	15	4.0	18.8*	7.4	27*	7.5
TATc (<4ng/ml)	29	66	#	-	460*	322	571	554

♣ Maximum mean values are shown.

* $p < 0.05$, versus baseline

No TATc responses occurred at a dose of 0.1 µg/kg rHuIL-12

Elevated thrombin-anti-thrombin III (TAT) complexes reflect activation of coagulative pathways. A coagulative response was not observed at 0.1 μg/kg rHuIL-12. However, at 0.5 μg/kg and 1.0 μg/kg rHuIL-12, half of patients had a coagulative response (table 1), with TAT complexes reaching mamimum values after a median of 12 hours (range: 4-72).

Table 1 shows maximum mean concentrations of PAP- and TAT complexes after different doses of rHuIL-12. The results of measurement of PAP- and TAT complexes of 19 patients that received 0.5 μg/kg rHIL-12 have been reported previously [Portielje et al, 2001]. Because the hemostatic mechanism is tightly linked to the inflammatory cascade, we now provide data from patients treated at other dose levels as well, in order to gain insight in the dose response relationship beween rHuIL-12 and parameters of systemic fibrinolysis and coagulation.

Cytokines

Plasma concentrations ol IL-12, TNF-α, IL-6 and IL-8 after IL-12 are shown in figure 4.

Blood samples for IL-12 measurements were available from 18 patients treated at dose level 0.5 μg/kg rHuIL-12. Serum IL-12 levels were below the detection limit in all patients before injection. After administration of rHuIL-12, plasma concentrations increased in 10 patients, with individual peak levels reached after 4 to 24 hours (median 12). Mean plasma IL-12 increased to 294 ± 218 pg/l. After administration of 0.5 μg/kg rHuIL-12, TNF-α levels increased in all patients, from a mean plasma concentration of 34 ± 20 pg/ml to a maximum of 93 ± 22 pg/ml ($p < 0.00001$). Baseline TNF-α concentrations were increased in 15 out of 19 patients with values between 22 and 83 pg/ml. Individual maximum levels were reached after 24 to 72 hours (median 48). The mean plasma concentration of IL-8 increased from 17 ± 18 pg/ml to a maximum of 34 ± 27 pg/ml ($p < 0.01$) at 48 hours. The mean concentration of IL-6 increased from 24 ± 28 pg/ml to a maximum of 84 ± 72 pg/ml ($p < 0.01$) at 12 hours.

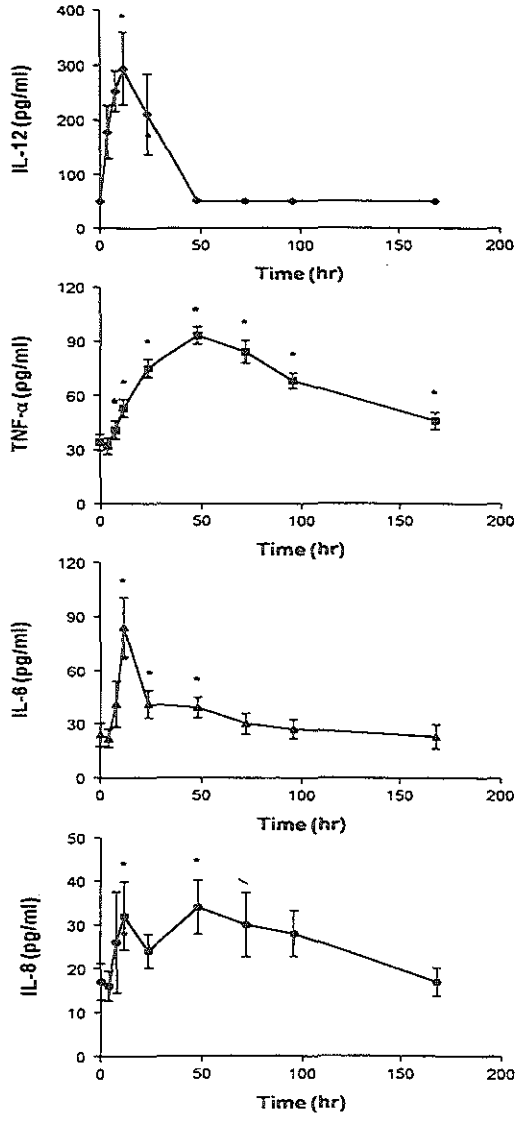

Figure 4. Plasma concentrations of IL-12, TNF-α, IL-6 and IL-8 after subcutaneous IL-12. *Mean ± standard error of the mean (SEM) plasma concentrations of rHuIL-12, TNFα, IL-6 and IL-8 during a period of 168 hours after a single subcutaneous injection of 0.5 μg/kg IL-12 in 19 patients with advanced renal cell carcinoma. Significant differences between baseline concentrations and concentrations after IL-12 injection are indicated by an asterix ($p<0.05$).*

Discussion

The present results demonstrate that s.c. IL-12 induces a dose dependent systemic activation of multiple inflammatory mediator systems in humans. IL-12 induced activation and degranulation of PMN in a dose dependent way in patients with advanced renal cell cancer. The activation of PMN was sustained, with ultimate normalization of plasma levels of degranulation products after 7 days. The effect of IL-12 on PMN degranulation is consistent with a study in chimpanzees that described PMN activation with maximum elastase-α1-antitrypsin complex concentrations at the last plasma sampling time-point of 48 hours [Lauw et al, 1999].

Since PMN express functional IL-12 receptors, degranulation may result from direct interaction with IL-12. This is supported by in vitro experiments showing that IL-12 upon binding increases intracellular free calcium, and induces actin polymerization, tyrosine phosphorylation and production of reactive oxygen radicals and platelet activating factor in PMN [Collison et al, 1998; Bussolati et al, 1998]. In our study, maximal concentrations of products of PMN degranulation in the peripheral blood coincided with peak levels of IL-12, i.e. at 24 hours post injection. In addition, IL-12 may activate PMN indirectly. We observed a dose-dependent induction of IL-6, IL-8 and TNF-α after IL-12 administration, and these cytokines all have known PMN-activating effects. However, our results suggest that PMN degranulation after IL-12 administration in vivo is probably not mediated by TNF-α or IL-8. In humans, TNF-α induces a very rapid degranulation of PMN, with maximum blood levels of elastase-α1-antitrypsin complexes and lactoferrin, 3 hours after TNF-α administration [Van der Poll et al, 1992]. In the present study maximum levels of both elastase-α1-antitrypsin complexes and lactoferrin were however reached at 24 hours, whilst TNF-α peaked at 48 hours, following injection of IL-12. Similarly, IL-8 is a strong PMN activator [Peveri et al 1994; Schroder et al, 1987; Downey, 1994] but again, peak plasma concentrations of IL-8 were observed 24 hours after levels of the degranulation products elastase-α1-antitrypsin complexes and lactoferrin reached their maximum. The assumption that IL-8 induction did not contribute to PMN degranulation is further supported by the observation that, whilst administration of IL-1β to humans induced elevation of IL-8 to levels similar to those observed in the present study, degranulation of PMN did not occur [Ogilvie et al, 1996]. IL-6 is another potential in vivo mediator of PMN activation after IL-12. PMN express IL-6 receptors [Keller et al, 1996] and IL-6 exposure has previously been shown to induce elastase and lactoferrin release and production

of platelet activating factor and oxygen-free radicals by PMN in vitro [Borish et al, 1989; Biffl et al, 1996]. In our study, peak levels of IL-6 at 12 hours post injection preceded peak levels of lactoferrin and elastase-α1-antitrypsin complexes at 24 hours post IL-12 injection. This result is consistent with the possibility that IL-6, formed in response to IL-12 injection in turn contributes to rhe release of elastase-α1-antitrypsin complexes and lactoferrin into the circulation. However, in a chimpanzee model of endotoxemia, in which IL-12 is an important mediator of the inflammatory response, PMN degranulation occurs independently of TNF-α, IL-8 and IL-6 synthesis [Kuipers et al, 1994]. These results indicate that IL-12 potentially activates PMN directly as well as indirectly.

IL-12 was also a powerful stimulus for the synthesis of the secretory phospholipase A_2 ($sPLA_2$), the enzyme that generates arachidonic acid and thus catalyzes the rate limiting step in the formation of lipid mediators. Endothelial cells, considered the most important source of $sPLA_2$ in the peripheral blood, have not been shown to express IL-12 receptors. IL-6 and TNF-α are the possible mediators of $sPLA_2$ synthesis after IL-12 injection, since either cytokine stimulates the production and release of $sPLA_2$ in-vitro by various cell types, including liver cells, endothelial cells and macrophages [Pfeilschifter et al, 1993; Redl et al 1993]. In healthy volunteers, TNF-α infusion resulted in increased $sPLA_2$, with maximum plasma levels after 6 hours [Van Dullemen et al, 1989]. Interestingly, the anti-tumor effect of TNF-α in a variety of tumor cell models depends on cytolysis that requires the activation of $sPLA_2$ by TNF-α [Mutch et al, 1992]. Furthermore, increased $sPLA_2$ synthesis after IL-12 may play an important role in inflammatory colitis, a life threatening side effect observed with systemic IL-12 administration [Leonard et al, 1997], as can be concluded from the observation that IL-12 does not cause gastrointestinal toxicity in a $sPLA_2$ deficient strain of mice [Car et al, 1999]. Presumably, this lack of toxicity in deficient mice is due to the lack of prostanoid contribution to tissue injury.

In accordance with previous clinical results [Atkins et al, 1997; Bajetta et al, 1998] we observed a rapid, IL-12-dose dependent, decrease of PMN in the peripheral circulation, with protracted depression of cell counts during the whole 7-day period of observation. Similar to humans, IL-12 administration to mice caused decreased numbers of circulating leucocytes and neutrophils [Eng et al, 1995; Romani et al, 1995]. The decreased PMN counts in the peripheral blood after IL-12 administration to humans is thought to occur due to compartmental cellular shift with accumulation of cells in liver, spleen and tumor sites. In vitro, IL-12 serves as a chemotactic stimulus for human PMN's [Allavena et al, 1994] and IL-12-induced platelet activating factor is thought to play a critical role in

this effect [Bussolati et al, 1998]. IL-12 potentially mediates PMN chemotaxis through the induction of TNF-α and IL-8. TNF-α upregulates adhesion molecules on endothelial cells and can mediate the migration of PMN's [Carlos et al, 1997; Bevilacqua et al, 1989; Pober et al, 1986]. In healthy individuals, intravenous TNF-α administration results in short-lived neutropenia, followed within an hour by a 24-hour lasting neutrophilia [Van de Poll et al, 1992]. IL-8 is a member of the α-chemokine family and an important PMN chemotactic factor [Schroder et al, 1987; Van Damme, 1991]. Intravenous injection of IL-8 in mice, resulted in an instant neutropenia, followed by profound neutrophilia for several hours [Pruijt et al, 1999]. The protracted elevation of TNF-α and IL-8 levels observed after IL-12 injection, i.e. lasting for more than 7 and more than 2 days, respectively, may well be responsible for the protracted neutropenia observed in our patients.

Clinical studies have addressed the systemic administration of IL-12 in patients with asthma, infectious diseases and cancer, but associated toxicity in combination with a lack of encouraging clinical results hamper further development of IL-12 in this line [Carreno et al, 2000; Hurteau et al, 2001; Bryan et al, 2000; Motzer et al, 2001]. More recently, the therapeutic application of IL-12 as an immune adjuvant in cancer and infectious diseases has received attention. Preclinical results have demonstrated that IL-12 provides a critical third signal along with antigen and IL-2 to activate CD8+ T cells [Curtsinger et al, 1999]. In addition, IL-12 has an adjuvant effect in the activation of CD8+ T cell responses to antigenic peptides in mice [Fallarino et al, 1999]. The immunological mechanisms underlying the adjuvant efficacy of IL-12 are incompletely understood. Here we show that locally injected IL-12 can activate PMN's which are thought to be engaged in a complex cross-talk, with immune and endothelial cells that bridges innate and adaptive immunity [Colombo et al, 1992; Di Carlo et al, 2001]. In particular, IL-12 has strong inflammatory effects in-vivo and may be a potent adjuvant by providing inflammatory signals which may optimize adequate antigen presentation. Indeed, most if not all, classical adjuvant substances promote local inflammation. However, the stimulation of excessive systemic inflammatory responses seems undesirable for an immune adjuvant. Although the number of patients treated at doses other than 0.5 μg/kg of IL-12 is, due to the original phase I design of the study, rather small, our results may give an indication of the dose of rHuIL-12 to be used as immune adjuvant in humans. At s.c. doses of 0.5 and 1.0 μg/kg, rHuIL-12 is a strong stimulator of systemic inflammatory mediator systems. However, after a dose of 0.1 μg/kg rHuIL-12, i.e. one fifth of the maximum tolerated dose, the systemic

inflammatory responses were only limited. Previously we have shown that at a dose of 0.5 μg/kg IL-12 induces activation of fibrinolysis and coagulation in humans [Portielje et al, 2001]. These side effects may have contributed to the hemorrhagic events, which complicated administration of IL-12 in other clinical phase I and II studies [Atkins et al, 1997; Motzer et al, 1998; Leonard et al, 1997]. In view of our current results that at 0.1 μg/kg rHuIL-12, PMN are activated, whilst only small amounts of systemic $sPLA_2$ are formed and activation of fibrinolysis is minimal, in the absence of activation of the coagulative response, we suggest that IL-12 as an adjuvant should not be used at doses higher than 0.1 μg per kg in order to prevent serious systemic inflammatory responses.

Chapter 6

IL-12, a promising adjuvant for cancer vaccination: a review

Adapted from:

IL-12, a promising adjuvant for cancer vaccination: a review

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Abstract

The clinical development of interleukin 12 (IL-12) as a single agent for systemic cancer therapy has been hindered by significant toxicity and disappointing anti-tumor effects. The lack of efficacy was accompanied by, and probably related to, declining biological effects of IL-12 in the course of repeated administrations at doses approaching the maximum tolerated dose (MTD).
Nevertheless, IL-12 remains a very promising immunotherapeutic agent because recent cancer vaccination studies in animal models and humans have demonstrated its powerful adjuvant properties. Therefore, IL-12 may re-enter the arena of cancer therapy.

Here we review the immune modulating characteristics of IL-12 considered responsible for the adjuvant effects, as well as the results of animal and human cancer vaccination studies with IL-12 applied as an adjuvant. In addition, we discuss how studies with systemic IL-12 in cancer patients, and several other lines of evidence indicate that IL-12 may exert optimal adjuvant effects only at low dose levels. Therefore, the MTD may not constitute the maximum effective dose of IL-12 for adjuvant application.

Introduction

Specific immunity against cancer, if present, is usually not effective, as shown by the course of most human cancers. Therefore, the discovery of tumor associated antigens for an increasing number of human malignancies [Boon et al, 1997; Rosenberg et al, 1999], has raised expectations of effective vaccination therapy of cancer, with the goal to induce immunity against cancer. Apparently, an effective immune response is not elicited by the tumor antigens that are expressed by cancers that have become clinically manifest. Indeed, in animal models, antigenic tumor cells have been shown to grow in immune-competent hosts without stimulating an acute or memory T cell response [Wick et al, 1997; Speiser et al, 1997]. An important role in the ineffective immune responses to cancer is thought to be played by the mechanism of immune tolerance. Some tumors are capable of in vitro tolerance induction in T lymphocytes that are specific for their tumor antigens [Staveley-O'Carroll et al, 1998]. The reversal of immune tolerance into immune activation may be one of the mechanisms by which cancer vaccination can become an effective treatment modality.

Among the strategies to stimulate an effective immune response against tumor antigens is the presentation of antigens together with an appropriate immune adjuvant. Recently, IL-12 has been identified as a powerful adjuvant substance in a variety of vaccination models of infectious disease. Promising results have also been obtained in animal cancer vaccination studies using either local or systemic co-administration of IL-12, or IL-12 gene-transduced cellular vaccines. The first results in humans clearly demonstrate that IL-12 enhances tumor specific cellular responses [Lee et al, 2001; Gajewski et al 2001]. IL-12 has several characteristics that seem essential for its adjuvant effects. In the vaccination area, IL-12 activates innate immune cells and promotes production of cytokines and chemokines, thereby mediating the attraction of other innate as well as specific immune cells to this region. We hypothesize that the co-administration of tumor antigens together with the strong pro-inflammatory cytokine IL-12, provides the environment with inflammatory danger signals required to activate antigen presenting dendritic cells (DC) and prevents tolerance induction towards the tumor antigens. In addition, IL-12 directs the development of T-helper lymphocytes towards the type 1 (Th1) functional profile that promotes cellular immune responses, and stimulates the proliferation of antigen specific cytotoxic T lymphocytes (CTL) and thereby establishment of immune memory.

The beneficial adjuvant properties of IL-12 that were demonstrated in infectious disease models [Gherardi et al, 2001], may not apply to tumor immunology. Various theoretical models of immune activation share the view, that tumor cells and infectious pathogens are differently recognized by the immune system. The "danger model" hypothesizes that specific immune activation, as opposed to tolerance, is initiated when innate immune cells recognize danger signals [Matzinger et al, 2001]. Invasion of pathogens is usually accompanied by local inflammation and tissue destruction, resulting in danger signals and activation of antigen presenting cells (APC) which then provide co-stimulatory signals to initiate specific immune responses. In contrast, when tumor antigens arise during malignant transformation, tissue destruction is initially minimal. The consequent absence of adequate danger signals is thought to result in immune tolerance towards the antigen. The "innate immune recognition model" assumes that specific immune responses are only activated when innate immune cells recognize conserved microbial structures with their pattern recognition receptors (PRR) [Medzhitov et al, 2000]. Once the PRR identify a pathogen-associated molecular pattern, the innate immune cells are triggered to perform their effector functions and activate specific immune cells. Tumor cells are unable to activate PRR, and hence do not trigger innate immune cells to activate specific immune cells. Because of these essential differences between the immune recognition of tumor cells and infectious pathogens, the present discussion is restricted to results obtained in experimental tumor models and cancer patients.

Molecular structure, production and cellular receptors

Interleukin 12 is composed of two disulfide-linked sub-units with molecular weights of 40kDa (p40) and 35kDa (p35) [Kobayashi et al, 1989; Podlaski et al, 1992]. The human p35 and p40 sub-units are structurally unrelated and have been mapped to chromosomes 3p12-3q13.2 and 5q31-q33 respectively [Sieburth et al, 1992]. Cells require co-expression of both genes to secrete biologically active IL-12 [Wolf et al, 1991]. IL-12 is primarily produced by phagocytic cells and antigen presenting cells (APC) such as monocytes, DC and activated B lymphocytes [Trinchieri et al, 1998; Heufler et al, 1996; Schultze et al, 1999], and production is strongly stimulated by infectious pathogens and their products [D'Andrea et al, 1992; Sato et al, 1996]. The other important

stimulus for IL-12 synthesis are interactions between CD40 and its ligand (CD154), on B cells or T cells and APC, respectively [Shu et al, 1995].

Natural killer- (NK) and T cells were first shown to express high affinity receptors for IL-12 composed of two sub-units, designated β1 and β2 [Presky et al, 1996]. Subsequently, several other cell types, such as neutrophils, DC, B lymphocytes and eosinophils, were shown to respond to IL-12 *in vitro* [Desai et al, 1992; Nutku et al, 2001; Nagayama et al, 2000; Vogel et al, 1996; Bussolati et al, 1998; Bryan et al, 2000; Airoldi et al, 2000]. Signal transduction through the high affinity receptors on lymphocytes involves tyrosine phosphorylation of the Tyk2 and Jak2 kinases and of the transcription factors STAT3 and STAT4 [Bacon et al, 1995a; Bacon et al, 1995b; Jacobson et al, 1995].

Clinical studies of systemic IL-12 as a single therapeutic anti-tumor agent

Recombinant human IL-12 has been studied as a single agent for systemic treatment in patients with various types of cancer. The development of IL-12 has proceeded along usual, FDA required lines, with initial phase I studies to determine tolerability and safety [Atkins et al, 1997; Motzer et al, 1998; Portielje et al, 1999; Gollob et al, 2000, Ohno et al, 2000] and subsequent efficacy studies [Leonard et al, 1997;Motzer et al, 2001; Hurteau et al, 2001]. The maximum tolerated dose (MTD) of IL-12, i.e., one dose level below the dose that caused dose limiting toxicity, was defined between 200 and 500 ng/kg,
in several intravenous (i.v.) and subcutaneous (s.c.) schedules consisting of 3 to 6 injections per 3 weeks. Common side effects consisted of fever and flu like symptoms, nausea, fatigue, mucositis and elevation of liver enzymes. IL-12 appeared to have an exceptionally long elimination half life, estimated between 9 to 25 hours, in comparison with other cytokines, [Portielje et al, 1999; Atkins et al, 1997; Motzer et al, 1998; Bajetta et al, 1998; Ohno et al, 2000]. A remarkable decrease of the area under the plasma concentration time curve (AUC) occurred after repeated injections of IL-12 [Portielje et al, 1999; Rakhit et al, 1999; Bajetta et al, 1998]. This reduction in AUC was possibly due to up-regulation of IL-12 receptors on lymphocytes in the course of treatment, in accordance with results obtained in a mouse model, and unrelated to anti-IL-12 antibody production [Thibodeaux et al, 1999].

The first phase II study unexpectedly resulted in severe toxicity and deaths. IL-12 was administered at the MTD defined in a previous phase I study [Atkins et

al, 1997], and the schedule was identical except for the omission of a treatment free period after the first dose [Leonard et al, 1997; Cohen, 1995]. Subsequent animal studies revealed that insertion of a treatment free period of a week after the first administration of IL-12, conform most phase I studies, reduced the toxicity of subsequent injections [Leonard et al, 1997; Sacco et al, 1997]. Moreover, these phase I studies also revealed that the reduction of side effects that occurred upon repeated injections [Portielje et al, 1999; Motzer et al, 1998] was accompanied by reduced IFN-γ induction [Sacco et al, 1997; Leonard et al, 1997; Bajetta et al, 1998; Portielje et al, 2002a; Rakhit et al, 1999; Gollob et al, 2000; Atkins et al, 1997]. In vitro, decreased IFN-γ secretion by T cells was related to cellular depletion of the signaling component STAT4 after prolonged IL-12 stimulation [Wang et al, 2001]. Since IFN-γ is considered to be the key regulator of IL-12 mediated anti-tumor effects, the down-regulation of its induction in the course of IL-12 treatment raised concerns [Brunda et al, 1995a; Nastala et al, 1994; Fujiwara et al, 1997;. In addition, our group showed that the down-regulation of biological effects also comprises the induction of TNF-α, IL-8 and IL-6 and the effect on leukocyte subset counts in the circulation. The concentrations of IL-10 remained elevated upon repeated IL-12 administrations [Portielje et al, 2002a; Bajetta et al, 1998; Ohno et al, 2000; Motzer et al, 2001]. It has been hypothesized that IL-10, as an endogenous counter regulator of many IL-12 mediated effects, is produced during IL-12 therapy to protect the body from the resultant ongoing and damaging inflammatory activity [Meyaard et al, 1996]. This hypothesis was supported by in vitro results demonstrating IL-12 to induce high levels of inhibitory IL-10 production by lymphocytes [Gerosa et al, 1996; Windhagen et al, 1996]. The results of Phase II studies, performed in patients with advanced renal cell and ovarian cancer, were disappointing, with overall response rates of only 7% and 4%, respectively [Motzer et al, 2001; Hurteau et al, 2001]. The lack of efficacy in these studies may be due to down-regulation of biological effects, including potential anti-tumor effects, due to endogenous IL-10 induction, that occurs at relatively high dose levels of IL-12.

In the development of IL-12 as a vaccine adjuvant, strategies that do not result in long-term systemic exposure to high concentrations of IL-12, such as administration of low doses or infrequent administrations, may therefore be necessary to prevent down-regulation of effects.

Properties of IL-12 that can mediate adjuvant effects

Effects on the innate immune system

Inflammation

As a strong pro-inflammatory cytokine, IL-12 induces the production of multiple other cytokines. Although the induction of IFN-γ predominates, it also enhances production of other pro-inflammatory cytokines such as GM-CSF, TNF-α, IL-8, IL-6, IL-15 and IL-18 in humans [Portielje et al, 2002a; Ohno et al, 2000; Gollob et al, 2000]. Importantly, IFN-γ operates in a positive feedback mechanism, as IFN-γ in turn stimulates IL-12 synthesis by phagocytic cells [Cassatella et al, 1995]. In accordance with previous results in non-human primates [Lauw et al, 1999], we have shown activation of multiple inflammatory mediator systems in patients with advanced renal cell cancer after s.c. IL-12 [Portielje et al, 2002b].

In vitro, binding of IL-12 to receptors on neutrophils results in activation of Ca2+ and tyrosine signaling pathways [Collison et al, 1998]. We also observed activation and degranulation of neutrophils in humans. The activation of neutrophils might be a prominent adjuvant property of IL-12, as neutrophils can operate as intermediates between innate and adaptive immunity, not only responding to cytokines, but also producing cytokines and chemokines that enable the attraction of other immune effector cells [Colombo et al, 1992; Ethuin et al, 2001]. For example, platelet activating factor, released from human neutrophils in response to IL-12, attracts other neutrophils and NK cells via chemotaxis [Bussolati et al, 1998]. The potentially important role played by neutrophils in cancer immune surveillance was demonstrated with tumor cells engineered to produce pro-inflammatory cytokines, such as IFN-γ and TNF-α. Vaccination with these cells resulted in anti-tumor immunity against wild-type parental tumor depending on neutrophils and CTL [Musiani et al, 1996].

Another inflammatory effect observed in humans after IL-12 administration, was an increase in serum concentrations of secretory phospholipase A_2 ($sPLA_2$) [Portielje et al, 2002b]. Secretory phospholipase A_2 may be released from endothelial cells in response to IL-12 mediated TNF-α and IL-6 synthesis, both known promotors of $sPLA_2$ production. This lipolytic enzyme releases fatty acids, often arachidonic acid, from membrane phospholipids [Crowl et al, 1991]. These are considered strong mediators of the inflammatory response.

Fibrinolysis and coagulation were also activated by IL-12 in humans [Portielje et al, 2001]. The coagulation system is integrally related to the innate immune

response and its activation promotes other inflammatory responses [Opal, 2000]. Thrombin formation occurred in 50% of patients after IL-12. Thrombin induces up-regulation of P-selectin and E-selectin; as a result aggregation of platelets with neutrophils, and interactions of neutrophils and monocytes with endothelial cells are promoted [Drake et al 1992; Anrather et al, 1997].

In conclusion, the pro-inflammatory properties of IL-12 can mediate activation and attraction of innate immune cells, resulting in the recruitment of specific immune cells.

IL-12 may also promote immune activation against tumor antigens because the activation of multiple inflammatory systems results in danger signals [Matzinger, 2001]. IL-12 production by phagocytic cells and APC is an early event shared by a variety of pathological states that evoke activation of the innate and, subsequently, antigen-specific immune responses [Sousa et al, 1997]. During sepsis and endotoxemia, IL-12 is produced [Heinzel et al, 1994; Jansen et al, 1996; Hazelzet et al, 1997], and the inflammatory response in these situations seems dependent on IL-12, as neutralizing antibodies against IL-12 can arrest the inflammatory cascade following bacterial lipopolysacharide administration [Wysocka et al, 1995]. Therefore, we propose, that co-administration of IL-12 and tumor antigens results in a local inflammatory response, with release of neutrophil elastase and other proteases, and synthesis of thrombin and lipid mediators, resulting in micro environmental damage [Weiss, 1989; Okrent et al, 1990; Kuipers et al, 1994]. This provides danger signals required for immune activation. As a result, local APC are activated, and can provide co-stimulatory signals and activate T cells.

Notably, stimulation of IL-12 production is also considered an important working mechanism in vaccination, whereby classical adjuvant substances exert their effects. This was shown for example for immune stimulating complexes containing saponin Quil A, and nucleic acid vaccines containing unmethylated CpG tracts [Smith et al, 1999; Sato et al, 1996; Chace et al, 1997].

Natural killer cells

NK lymphocytes are also activated by IL-12, in fact, it was initially discovered in 1989 as NK cell stimulatory factor [Kobayashi et al, 1989]. IL-12 promotes NK cell cytotoxicity, cytokine production, in particular high levels of IFN-γ [Aste-Amegaza et al, 1994; Naume et al, 1992; Robertson et al, 1992], and mediates NK cell chemotaxis [Allavena et al, 1994; Fogler et al, 1998]. In cancer patients, IL-12 indeed enhances the cytolytic activity of NK cells and increases the expression of CD2, LFA-1 and CD56 molecules, that mediate NK

cell migration [Robertson et al, 1999]. In a mice Leishmania infection model, NK cells were shown to exert an intermediate function between the innate and specific immune responses. The strong Th1 response, obtained after administration of leishmanial antigens in combination with IL-12 to mice, depended on NK cells and could be completely abrogated by in vivo depletion of NK cells [Afonso et al, 1994]. NK cells also have direct cytotoxic effects against MHC class I deficient tumors [Trinchieri, 1989]. Tumor eradication after vaccinations supported by adjuvant IL-12 is dependent on NK cells in several animal models [Jyothi et al, 2000; Smyth et al, 2001; Kodama et al, 1999]. In this context it is of interest that IL-12 deficient mice are more sensitive to chemical carcinogens and develop increased numbers of metastases following injection of transplantable tumor, as compared to wild-type controls, and that this immune surveillance defect is related to sub-optimal NK-cell function [Smyth et al, 2000].

Dendritic cells

Moreover, IL-12 enhances the function of DC, which are professional APC capable to process antigen in the setting of vaccination, as they provide high concentrations of peptide /MHC ligands for T cell receptor engagement, required to activate specific immunity. DC express IL-12 receptors, and their occupation initiates nuclear localization of members of the NF-κB family of transcription factors [Grohmann et al, 1998]. They are supposed to increase the maturation of DC and enhance their capability to present antigen [Pettit et al, 1997], e.g., by up-regulation of class II MHC expression [Grohmann et al, 2000]. Furthermore, IL-12 promotes the differentiation and maturation of DC indirectly, via the induction of pro-inflammatory cytokines. The pro-inflammatory cytokines TNF-α, IL-6, and GM-CSF have been shown to mediate migration of DC to T-cell rich areas of lymphoid organs in order to form clusters with antigen-specific T cells, creating the appropriate environment for T helper cell differentiation [Austyn, 1996; Jonuleit et al, 1997]. In addition, IFN-γ enhances antigen processing by DC and their MHC class I-presentation of antigen [Brossart et al, 1997; Früh et al, 1999].

In recent years, DC based vaccines have received intense interest [Reid, 1998]. DC can be generated in vitro [Young, 1999] and loaded with tumor cell lysates or tumor peptides before administration to the patient. In this way the physiological process that recruits antigen specific T cells is mimicked to some degree. Although mature DC themselves are potent producers of IL-12, co-

administration of IL-12 improves the results of DC based vaccines. In vitro, CTL responses, triggered by autologous human monocyte-derived DC that were modified to express melanoma antigens, could be enhanced by co-transfecting these DC with IL-12 genes [Tüting et al, 1998]. In situations where antigen presentation by DC without IL-12 co-administration induced T cell anergy, IL-12 could reverse or prevent development of tolerance in favor of immune activation [Grohmann et al, 1997]. Similarly, IL-12 was shown to be able to convert DC from a state of tolerance to activity. In patients that presented simultaneously with progressing and regressing metastases, tissue DC from progressing metastases appeared unable to induce T cell proliferation and did not produce Th1 cytokines, in contrast to DC from regressing metastases, and this defect could be overcome by IL-12 addition [Enk et al, 1997].

In conclusion, IL-12 plays an important role in the activation of innate immunity and potentially provides tumor antigens with a background of inflammatory effects with resultant "danger" signals that can promote activation of specific immunity.

Effects on specific immune cells

Cellular immune response

The cytokines present in the micro-environment at the time of initial antigen stimulation direct the differentiation of naive T cells into effector T cell subsets. MHC-restricted, Ag-specific T lymphocytes are considered to be an important effector mechanism against cancer.

In the presence of IL-12 naive T cells differentiate into the functionally defined Th1 subset [Hsieh et al, 1993] that is involved in cell mediated immunity. Subsequently, IL-12 is an important co-stimulus for proliferation and further activation of fully differentiated Th1 cells and their secretion of IFN-γ [Manetti et al, 1993; Germann et al, 1993].

T lymphocytes respond to IL-12 through high affinity receptors, which are composed of two sub-units, termed β1 and β2 [Presky et al, 1996]. Th1 cells express both sub-units. However, if T helper cells differentiate along the Th2 pathway, supporting humoral immune responses, they selectively lose IL-12Rβ2 and thereby become unresponsive to IL-12 [Szabo et al, 1997]. Th1 commitment is enhanced by IFN-γ, which further up-regulates the IL-12 receptor [O'Garra, 1998; Trinchieri, 1996]. Once a Th1 response is induced in vivo, IL-12 is in most cases not necessary for maintaining this response [Gazzinelli et al, 1994]. This observation is important for vaccination strategies, as it implies that addition of

IL-12 to the vaccine only would be sufficient to induce and maintain the desired response.

In addition, IL-12 modulates a number of genes involved in Th1 trafficking and regulates the migration and homing of these cells. IL-12 can attract and maintain Th1 cells to the site of administration by the up-regulation of Th1 specific adhesion molecules and their ligands. For example, IL-12 selectively increases the expression of integrin-α6/β1 and chemokine receptor CCR1 on Th1 cells in vitro [Colantonio et al, 1999]. Also, IL-12 upregulates the expression of glucosyltransferase enzymes that increase the expression of P-selectin and E selectin ligand on Th1 cells which enables their recruitment to inflamed tissues [White et al, 2001]. Finally, IL-12 strongly induces the expression of IP-10 in various cell types in vitro [Sgadari et al, 1996]. IP-10 is the ligand of chemokine receptor CXCR3, selectively expressed on Th1 cells [Mantovani, 1999]. In accordance, peripheral blood mononuclear cells [PBMC) and tumor biopsies from cancer patients showed increased expression of IP-10 after IL-12 treatment [Haicheur et al, 2000; Bukowski et al, 1999].

DC play an essential intermediate function in the facilitating interaction between T helper cells and antigen specific cytotoxic CD8+ T lymphocytes (CTL). Priming of CTL is enabled by the ligation of CD40 on DC and its ligand CD154 on activated CD4+ cells [Schoenberger et al, 1998; Bennett et al, 1998]. The strong induction of IL-12 synthesis that occurs as a result of CD40 ligation, suggests an important role for IL-12 in the molecular mechanisms responsible for the CTL priming. This contention is further supported by studies using latex microspheres coated with various combinations of class I MHC-peptide complexes and co-stimulatory molecules, thus avoiding the use of APCs whose function may be affected by cytokines. It was hence shown that IL-12, in the presence of antigen, acts directly on the naive CD8+ CTL to promote clonal expansion and differentiation [Curtsinger et al, 1999].

That IL-12 plays an important role in the establishment of immunological memory was demonstrated in an experimental system, in which a small number of antigen-specific CD8+ CTL were adoptively transferred into naive, syngeneic mice, in order to monitor responses to peptide immunization in the absence or presence of IL-12. Peptide immunization without simultaneous IL-12 administration induced a weak and transient expansion of CD8+ CTL, whereas in the presence of IL-12, a large clonal expansion of CD8+ T cells was induced in the draining lymph nodes. These cells were capable of antigen specific killing in in vitro assays. Additionally, a stable memory T cell population was generated that responded to a second challenge with IL-12 and peptide [Schmidt et al,

1999]. A strong specific CTL response was observed in patients with advanced melanoma after administration of IL-12. Numbers of tumor specific CTL increased in the circulation and influx of specific memory CD8+ T cells into metastasized lesions was documented [Mortarini et al, 2000].

Humoral immune response

With respect to humoral immunity, addition of IL-12 to protein and hapten vaccinations strongly up-regulates the synthesis of Ag-specific, complement fixing IgG2a, IgG2b and IgG3 antibody subclasses [Germann et al, 1995; Buchanan et al, 1995]. Further experiments in mice revealed that the elevation of these antibody isotypes is dependent on IFN-γ induction [Metzger et al, 1997]. However, in IFN-γ knock-out mice, IL-12 still significantly enhances the synthesis of specific IgG1 and IgG2b. Therefore a two step model of humoral immune enhancement by IL-12 was proposed [Metzger et al, 1997]. Initially, the IL-12 induced production of IFN-γ by Th1 and NK cells, would mediate early switching of B cells towards IgG2 immunoglobulin secretion with temporal suppression of IgG1 production. Subsequently, IL-12 would stimulate the switched B cells to secrete increased amounts of antibody, regardless of their isotype [Metzger et al, 1996].

IL-12 was also identified as a pivotal molecule secreted by activated human DC that promote the differentiation of naive B cells into IgM secreting plasma cells and hence plays an important role in generation of primary antibody responses that are initiated by DC [Dubois et al, 1998]. Finally, IL-12 may exert indirect effects on B cells via the induction of other cytokines than IFN-γ. We have shown in patients with renal cell cancer, that IL-12 induces elevation of serum levels of IL-6 [Portielje et al, 2002a], which is a prominent stimulator of B cell differentiation and immunoglobulin synthesis [Van Snick, 1990].

Adjuvant effects of IL-12 in animal studies

The addition of IL-12 to different types of cancer vaccines has been extensively studied in animal (mostly murine-) models. The first vaccination protocol addressed the co-administration of IL-12 with tumor derived peptide and resulted in the induction of peptide specific CTL in naive, tumor bearing mice and the eradication of established tumors [Noguchi et al, 1995]. Several studies used cancer cells as vaccines, that had been transduced to express IL-12 [Tahara et al, 1995 and 1996; Rodolfo et al, 1996; Fallarino et al, 1997 and 1999;

Schmitt et al, 1997; Cavallo et al, 1997; Chen et al, 1997; Popovic et al, 1998; Hoshino et al, 1998, Lode et al, 1998, Myers et al, 1998; Fuji et al, 1999; Dunussi-Joannopoulos et al, 1999; Carr-Brendel et al, 1999; Adris et al, 2000; Lechanteur et al, 2000, Nagai et al, 2000]. Alternative approaches also resulted in the presence of IL-12 at the site of tumor antigen. Recombinant viral vectors, encoding IL-12 [Bramson et al, 1996] Caruso et al, 1996; Puissieux et al, 1998; Rakhmilevich et al, 1997; Fernandez et al, 1999], or fibroblasts, transfected for IL-12 production, were injected near the tumor [Tahara et al, 1994; Matsumoto et al, 1999]. More recently, studies have applied co-administration of genes encoding for IL-12 and various tumor antigens [Tan et al, 1999; Amici et al, 2000; Song et al, 2000; Kim et al, 2001]. The addition of IL-12 to these vaccines clearly enhanced the anti-tumor effects, with resultant inhibition of tumor growth and eradication of established tumors. Additionally, immune memory was established with rejection of tumor cells at a subsequent challenge. In several studies, separate analyses have demonstrated that IL-12 plus vaccine was more effective than either component alone [Pulaski et al, 2000; Rodolfo et al, 1996; Lechanteur et al, 2000; Fuji et al, 1999; Hoshino et al, 1998; Jean et al, 1998; Adris et al, 2000; Cavallo et al, 1997; Dunussi-Joannopoulos et al, 1999; Fallarino et al, 1997 and 1999]. In vivo depletion of cellular subsets [Adris et al, 2000; Dunussi-Joannopoulos et al, 1999; Lode et al, 1998; Musiani et al, 1996; Pulaski et al, 2000; Weber et al, 1999; Puisieux et al, 1998] and knockout mice [Hunter et al, 1997; Puisieux et al, 1998; Song et al, 2000] have been used to investigate the anti-tumor mechanism of IL-12. Additionally, the cellular infiltrate in tumor metastases after vaccination has been characterized [Hunter et al, 1997; Fernandez et al, 1999; Nanni et al, 2001]. In most studies, lymphocytes were pivotal effector cells. The lymphocyte subsets involved, such as CD 8+ T cells, CD4+ T cells, NK cells or a combination of these, varied with the specific vaccine and the tumor model studied. Infrequently, other immune effector cells such as macrophages have been implicated in the anti-tumor effects of IL-12 [Tsung et al, 1997]. Additionally, Il-12 was shown to stimulate humoral immunity. In a model of colon carcinoma, vaccination with IL-12 transduced tumor cells cured 40% of tumor bearing mice. Favorable anti-tumor responses were related to the synthesis of antibodies against tumor associated antigens that induced tumor cell lysis in a complement dependent cytotoxicity assay [Rodolfo et al, 1996]. Moreover, IL-12 increased anti-neu antibody synthesis, in a model in HER-2/neu transgenic mice. Although antibody levels were not correlated with anti-tumor protection, vaccination with a combination of plasmids

encoding the neu oncogene and IL-12, resulted in protection against mammary tumors that normally develop spontaneously in these mice [Amici et al, 2000].

In several recent studies IL-12 has been combined with other strategies aimed to promote effective immune responses against tumor antigens. The administration was systemically, together with tumor cells transduced to express co-stimulatory molecules such as B7-1 or MHC class II [Joki et al, 1999; Pulaski et al, 2000]. An important and promising vaccination strategy consists of the addition of IL-12 to DC based vaccines [Melero et al, 1999; Zitvogel et al, 1996; Fallarino et al, 1999; Koido et al, 2000]. In a mouse model of chemically induced fibrosarcoma, DC were pulsed with tumor peptides, that had been eluted with acid from autologous tumor [Zitvogel et al, 1996]. These DC were combined with intra-peritoneal administration of IL-12. Alternatively, antigen-loaded DC were transfected with a retroviral vector or a pro-viral construct encoding murine rIL-12. Both strategies augmented the anti-tumor effect of the vaccine, enhanced the growth arrest of established tumors and increased specific cytotoxicity of splenic T cells, as compared to treatment with non-transfected, peptide pulsed DC or IL-12 alone. A recent experiment with DC in MHC-1 transgenic mice demonstrated that IL-12 can even reverse tolerance in vivo. MUC1 is over-expressed in human breast and other cancers. Administration of MUC-1 expressing DC to the MUC-1-transgenic mice only elicited a specific anti-MUC immune response, if IL-12 was co-administered along with the DC [Koido et al, 2000]. MUC-1-specific CTL were also induced when antigen pulsed PBMC, instead of DC, served as APC. Because peptide-loaded autologous human PBMC can be obtained relatively easy, in contrast to DC, this is an attractive approach to translate for clinical use and indeed, similar studies are now performed in humans.

Adjuvant effects of IL-12 in human studies

Clinical experience in humans is still limited. Results of two studies with tumor peptide vaccination and IL-12 co-administration in patients with malignant melanoma were recently published [Gajewski et al, 2001; Lee et al, 2001]. One study was performed in patients with metastasized melanoma, using a vaccine consisting of autologous PBMC pulsed with MAGE-3 or MelanA peptides and co-administration of recombinant IL-12 [Gajewski et al, 2001]. Fifteen HLA-A2 positive patients with metastases expressing MAGE-3 or Melan-A, were vaccinated with these tumor peptides at least 3 times at three week intervals.

Different doses of IL-12 were used (0, 30, 100 or 300 ng/kg) and IL-12 was administered s.c., adjacent to the vaccination site, on days 1, 3 and 5. Only one out of four patients treated with pulsed PBMC without IL-12, but all patients treated with 30 or 100 ng/kg of IL-12 developed a specific CD8+ T cells response after 3 immunizations. Remarkably, only one out of three patients treated at the highest dose level of 300 ng/kg of IL-12 did so. Furthermore, grade 2 or 3 toxicity (fatigue, depression and decreased numbers of peripheral blood cells) only occurred with the highest dose of IL-12. Most importantly, 6 of 8 patients with tumor-specific CD8+ T cells showed regression of all, or part of their metastases. In the second study, patients with stage III or IV malignant melanoma, who had undergone complete resection of macroscopic tumor, were vaccinated with peptides derived from the tumor antigens gp100 and tyrosinase, emulsified with incomplete Freund's adjuvant [Lee et al, 2001]. Patients received, during 26 weeks, a total of 8 vaccinations, with or without 30 ng /kg IL-12. The combination augmented gp 100-specific DTH reactivity and boosted the gp100- and tyrosinase specific production of IFN-γ by peripheral blood T cells after repeated vaccinations. The number of gp100 specific CTL as measured by tetramer flow cytometry was also augmented by IL-12. Of note, the generation of specific CTL responses took several vaccinations over multiple months. This observation confirmed the clinical impression that patients with rapidly progressive disease may not benefit from therapeutic vaccination. Time to relapse was not influenced by the addition of IL-12 to the regimen, and did not correlate with any of the immunological results. In a third study, the treatment of 6 patients with advanced melanoma with weekly vaccinations, using IL-12 gene-transfected autologous irradiated tumor cells, resulted in one mixed response (disappearance of part of the metastases). Two of 6 patients had an increased specific CTL response, as measured 2 weeks after the third vaccination, one of whom had a mixed response the other stable disease [Möller et al, 2000]. In this study, lymphokine activated killer cell activity was induced in the majority of patients, but was not related to clinical outcome. In another clinical protocol, peritumoral injection of IL-12 transfected fibroblasts was shown feasible, and reduction of tumor masses near the injection site were observed [Kang et al, 2001].

Inverse Dose response effect

In a human study that bears similarities to the study by Lee, patients with advanced malignant melanoma were treated with a vaccine consisting of gp100 melanoma tumor antigen in incomplete Freund's adjuvant [Rosenberg et al, 1999]. The vaccine elicited the generation of anti-peptide and anti-tumor T cell precursors in the circulation, while 42% of patients exhibited objective tumor regression, but unfortunately without attaining a clinical response. In sharp contrast with results by Lee, co-administration of IL-12 reduced the number of T cell precursors and anti-tumor responses were no longer observed. However, IL-12 was administered i.v. at a relatively high dose level, i.e. 250 ng/kg/day, on 5 consecutive days after each peptide vaccination.

In analogy with clinical studies of systemic single agent IL-12 administration, the biological effects of IL-12 may be down-regulated at the higher dose levels. Additional lines of evidence indicate that IL-12 in the setting of vaccination studies, does not exerts optimal immune modulation at high dose levels, and that above a certain threshold level, the dose response relationship may revert [Noguchi et al, 1995; Jean et al, 1998; Kurzawa et al, 1998]. A very low dose of 1 ng per day, eight times during 2 weeks, co-administered with a p53 tumor peptide vaccine, induced tumor rejection and CTL generation in a murine sarcoma model, whereas doses higher than 10 ng per day failed to do so [Noguchi). In a rat glioma model, vaccination with irradiated tumor cells in combination with subcutaneous IL-12, resulted in maximal tumor eradication and optimal protective immunity against repeated tumor challenge at the lowest applied dose of 1 ng of IL-12 per day, for 28 days [Jean et al, 1998]. In contrast, treatment with high doses of 250 ng per day, for 10 days, prevented the generation of tumor specific CTL induced by immunization with GM-CSF-transfected tumor cells [Kurzawa et al, 1998]. A possible inhibitory role for IL-10 has been suggested in a model of adoptive transfer of specific CTL in immuno-deficient mice bearing autologous tumor. In these mice, tumor growth suppression by CTL increased after injection of 100 ng of IL-12 into the tumor, every 2 to 3 days, but not after higher doses of 1000 ng [Asselin-Paturel et al, 2001]. In vitro, it was then shown, that high dose IL-12 stimulated, in addition to IFN-γ-, the production of high levels of IL-10 from the tumor specific CTL, and furthermore, anti-IL-10 polyclonal antibodies could abrogate the inhibition of tumor cell lysis observed after high dose IL-12. Since IL-12 and IL-10 have opposite effects on the accessory function of DC and other APC [Koch et al, 1996], dose finding studies for IL-12 as adjuvant in therapeutic anti-tumor

vaccination should focus on those dose levels that do not induce IL-10 production.

Conclusions

IL-12 is a promising adjuvant for cancer vaccination and has the potential to activate an effective immunological response to cancer. Firstly, it has strong inflammatory properties, and causes the induction of other pro-inflammatory cytokines, degranulation of neutrophils, the formation of lipid mediators and activation of the coagulative and fibrinolytic systems, that together can provide an environment of multiple danger signals for tumor antigens, suitable for the activation of professional APC. In addition, IL-12 directly and indirectly activates innate immune effector cells such as neutrophils and NK cells, and promotes their secretion of substances that alter the microenvironment and promote expression of adhesion molecules that mediate trafficking and homing of APC and specific immune cells. Moreover, IL-12 enhances the maturation and antigen presenting efficacy of DC and promotes T helper cell differentiation towards Th1 type, necessary for cellular immune responses. Finally, it stimulates the differentiation and lytic capacity of antigen specific CTL and promotes immune memory.

The strong adjuvant properties of IL-12 have been demonstrated in a variety of animal models using different vaccination strategies that united tumor antigens and IL-12. Sophisticated vaccines have been constructed with antigen pulsed DC or PBMC, transduced to express increased IL-12. In these animal cancer models, IL-12 was shown to clearly enhance eradication of established tumor and moreover was capable of inducing a specific anti-tumor immune memory. The first human studies addressing the co-administration of systemic IL-12 to cancer vaccines have shown development of tumor-specific CTL in the course of multiple vaccinations and although clinical responses were limited, CTL responses were clearly correlated with clinical tumor regressions. Several lines of evidence indicate that the optimal immune regulatory effects of IL-12 are confined to the lower dose levels at which the induction of IL-10 does not take place. The maximally effective dose, schedule and route of administration remain to be defined.

Based on the reviewed data, we anticipate the revival of IL-12 as adjuvant for therapeutic vaccination against cancer.

Chapter 7

Summary

The immunotherapy of cancer is based on the assumption that the immune system can be stimulated to recognize cancer and eradicate tumor. One of the approaches of immunotherapy consists of systemic administration of cytokines. Interleukin 12 (IL-12) is a cytokine with important immunoregulatory functions. The encouraging anti-tumor effects, observed in a variety of animal tumor models, have resulted in the development of IL-12 as a single agent for systemic cytokine therapy of cancer in humans. Renal cell cancer is one of the few human cancers that are more responsive to immunotherapy than to conventional cytotoxic therapies.

We therefore performed a phase I and pharmacokinetic study of subcutaneously administered IL-12 in 28 patients with advanced renal cell cancer (chapter 1). Recombinant human IL-12 was administered on day 1, followed by an observation period of 7 days. Starting on day 8, repeated injections were administered 3 times a week, for two weeks. Dose limiting toxicity (DLT) of the initial injection was observed at 1.0 μg/kg and consisted of fever, peri-vasculitis of the skin and leukopenia. DLT of the subsequent repeated injections was observed at 1.25 μg/kg and comprised deterioration of performance status, fever, vomiting, mental depression and leukopenia. Other notable toxicity consisted of oral mucositis and elevation of hepatic enzymes. Fever, leukopenia and elevation of hepatic enzymes were more severe after the initial injection than after repeated injections at the same dose level. Peak levels of IL-12 were reached 9.7 hours after administration and the mean elimination half-life was 9.4 hours, which is exceptionally long for a cytokine. At dose level 0.5 μg/kg, the mean area under the plasma concentration-time curve (AUC) significantly decreased from 7.4 ng x h/ml after the first injection to 3.3 ng x h/ml after repeated administrations, and at dose level 1.0 μg/kg, from 32 ng x h/ml to 6.0 ng x h/ml, possibly due to upregulation of IL-12 receptors. It was concluded that the maximum tolerated dose (MTD) of the initial injection of rHuIL-12 was 0.5 μg/kg, whereas the MTD of 3 subsequent administrations per week was, 1.0 μg/kg.

Upon repeated administrations of IL-12, side effects diminished. This prompted us to study whether or not the immunological effects decreased as well (chapter 3). Serial blood samples were collected from 26 patients in the course of the phase I study. The first injection of IL-12 was accompanied by rapid, transient and dose dependent increments of plasma levels of IFN-γ, TNF-α, IL-10, IL-6, IL-8, but not IL-4. Additionally, rapid, transient, and dose dependent reductions of lymphocyte-, monocyte- and neutrophil counts were observed in the peripheral blood. The major lymphocyte subsets, i.e. CD 4+ and CD8+ T cells, B cells and NK cells, followed this pattern. Upon repeated injections,

concentrations of the inhibitory cytokine IL-10 further increased, while increments of plasma levels of IFN-γ, TNF-α, IL-6 and IL-8, as well as fluctuations of leukocyte subset counts, were tapered. Dose escalation of IL-12 within clinically tolerable margins did not reduce the decline of these immunological effects. Thus, upon repeated administrations of IL-12, levels of pro-inflammatory cytokines diminish, as well as effects on peripheral blood leukocyte subsets, while IL-10 production increases and may mediate the observed down-regulation of clinical and immunological effects.

Several bleeding episodes that have been reported in the literature as a result of IL-12 administration to humans prompted us to study the effects of IL-12 on fibrinolysis and coagulation (chapter 4). Coagulative and fibrinolytic responses were assessed in 18 patients with advanced renal cell carcinoma after subcutaneous administration of 0.5 μg/kg rHuIL-12. IL-12 induced sustained activation of fibrinolysis in the majority (94%) and substantial thrombin generation in half of patients. Plasmin-α2-antiplasmin complexes (PAPc) increased from 12 to a maximum of 19 nmol/l at 72 hours. IL-12 induced the production of tissue plasminogen activator (tPA), whereas secretion of its natural inhibitor plasminogen-activator inhibitor-I (PAI) diminished. The elevation of tPA and reduction of PAI correlated with elevation of PAPc. Thrombin-anti-thrombin III complexes increased from 29 to a maximum of 460 ng/ml at 12 hours. The fibrinolytic response continued while thrombin generation had already abated and therefore occurred independently. Sequence and time frame of fibrinolytic and coagulative responses differ markedly from those observed after other cytokines and are not in agreement with an important mediator function of IFN-γ or TNF-α.

Studies in experimental animals and humans have shown that endogenously produced IL-12 plays an important role in the toxic sequel of sepsis and endotoxemia. In these situations, excessive activation of various components of the inflammatory cascade contributes to the development of tissue injury and mortality. We therefore studied the in-vivo effects of different doses of IL-12 on components of the inflammatory cascade (chapter 5). A single dose of 0.5 μg/kg IL-12 induced significant degranulation of neutrophils: plasma levels of elastase (azurophilic granules) and lactoferrin (specific granules) increased from 48 to 117 ng/ml and from 124 to 181 ng/ml, respectively, at 24 hours. Additionally, IL-12 injection mediated the release of lipid mediators: Plasma concentrations of secretory phospholipase A2 (sPLA2) increased from 17 to 97 ng/ml. Systemic activation of inflammation by IL-12 occurred in a dose dependent way. At 0.1 μg/kg of IL-12, systemic responses were minimal: Although mild activation of

neutrophils was already detectable, formation of sPLA2 was limited. Because the hemostatic mechanism is tightly linked to the inflammatory cascade, it is important to note, that at this dose level, there was neither activation of the coagulative response and only minimal activation of fibrinolysis.

While performing the studies presented in this thesis, reports of the first phase II studies of single agent systemic IL-12 in patients with advanced ovarian and renal cell cancer were published and demonstrated disappointing anti-tumor efficacy. In these studies, IL-12 was used at the MTD as defined in previous phase I studies, including the study described in chapter 2. Probably, the lack of efficacy is related to the decline of immunological effects of IL-12 that occurs in the course of repeated administrations at the MTD. Protective feedback mechanisms, such as the production of IL-10, may operate to abrogate the strong and potentially damaging systemic inflammatory responses that occur after relatively high dose IL-12.

Recent cancer vaccination studies in animal models and humans have demonstrated that IL-12 has powerful adjuvant properties and hence remains a promising immunotherapeutic agent. All classical adjuvant substances are strong stimulators of local inflammation and therefore, the stimulation of inflammatory responses is thought to play an important role in the adjuvant effects of IL-12. In chapter 6, the adjuvant properties of IL-12 are reviewed. It is described how the pro-inflammatory effects of IL-12 can provide an environment of multiple danger signals for tumor antigens, resulting in activation of professional antigen presenting cells. Results of animal and human studies with IL-12 applied as an adjuvant for cancer vaccination are reviewed. Finally, it is discussed how clinical studies with systemic IL-12 and several other lines of evidence indicate that IL-12 may exert optimal adjuvant effects only at low dose levels, and consequently, that the maximum tolerated dose is not be the most effective dose of IL-12.

Final conclusions and future perspectives

The initial administration of IL-12 to humans induces transient elevation of various other pro-inflammatory cytokines, and transient depletion of leukocytes in the peripheral blood. Upon repeated administration of IL-12, the induction of the inhibitory cytokine IL-10 is sustained, whereas the other immunological effects diminish. The disappointing anti-tumor effects observed in the first phase II studies are possibly related to this decline of biological effects. However, IL-12 may re-enter the arena of cancer therapy, because recent cancer vaccination

studies have shown that IL-12 is a promising adjuvant substance, that seems to exert optimal effects at low dose levels. We hypothesize that localized inflammation plays a key role in the adjuvant effects of IL-12. On the other hand, administration of high dose IL-12 will trigger endogenous feedback mechanisms that annihilate the potential therapeutic effects. We anticipate therapeutic application of IL-12 as adjuvant for vaccination against cancer.

Chapter 8

Samenvatting

Het onderzoek dat wordt beschreven in dit proefschrift bestudeerde de klinische en biologische effecten van interleukine 12 (IL-12) bij patiënten met niercelkanker.

Inleiding

Niercelkanker is een relatief zeldzame ziekte. Patiënten met deze vorm van kanker hebben in het algemeen een slechte prognose, omdat veel van hen bij presentatie van de ziekte al uitzaaiingen hebben. Helaas is uitgezaaide niercelkanker meestal niet goed te behandelen met chirurgie, radiotherapie of chemotherapie. Daarom wordt er veel onderzoek gedaan naar nieuwe manieren van behandeling.

Het concept van immunotherapie bij kanker is gebaseerd op de veronderstelling dat het immuunsysteem kan worden gestimuleerd om kanker cellen te herkennen en te vernietigen. Eerder onderzoek heeft aangetoond dat niercel kanker een van de zeldzame kanker soorten is die relatief gevoelig is voor immunotherapie. Hoewel slechts een klein gedeelte van de patiënten met uitgezaaide niercelkanker baat heeft bij de nu toegepaste vormen van immunotherapie, genezen sommige patiënten uiteindelijk geheel. Dit resultaat motiveert onderzoek naar nieuwe immunotherapeutische behandelingen bij patiënten met uitgezaaide niercelkanker. Eén van de vormen van immunotherapie bestaat uit het toedienen van cytokines. Cytokines zijn in het lichaam natuurlijk voorkomende stoffen, die geproduceerd worden door verschillende cellen van het immuunsysteem en vervolgens de werking van andere immuuncellen sturen. In dierproeven werd aangetoond dat het cytokine IL-12 sterke anti-tumor effecten heeft.

Het onderzoek

Allereerst deden we een fase I onderzoek met IL-12 bij patiënten met uitgezaaide niercelkanker (hoofdstuk 2). In fase I onderzoek wordt bepaald hoe een potentieel geneesmiddel door patiënten wordt verdragen. Dit type onderzoek gaat vooraf aan fase II onderzoek, waarmee de uiteindelijke werkzaamheid bij een bepaalde ziekte wordt bepaald. Fase 1 onderzoek is niet erg geschikt om de werkzaamheid van potentiële geneesmiddelen te bepalen, omdat uiteindelijk

maar een klein aantal mensen met de meest effectieve dosis van het middel wordt behandeld.

In ons onderzoek werd IL-12 met onderhuidse injecties, in een langzaam opklimmende dosering, aan kleine groepen patiënten gegeven waarbij nauwkeurig werd bepaald wat de bijwerkingen bij de verschillende doseringen waren. Het onderzoek werd uitgevoerd in de Dr. Daniel den Hoed Kliniek, behorend tot het Erasmus Medisch Centrum in Rotterdam en in de Johannes Gutenberg Klinik, behorend tot de universiteit van Mainz in Duitsland. Met de studie werd bepaald welke de hoogste dosis is die kan worden toegediend zonder belangrijke bijwerkingen. Deze dosis was vervolgens de geadviseerde dosis voor fase II onderzoek dat in andere centra werd verricht. Frequente bijwerkingen van IL-12 waren: koorts, vermoeidheid, afname van de witte bloedcellen en verhoging van de leverenzymen in het bloed. In de loop van de behandeling kon de toegediende dosis IL-12 worden verhoogd, omdat de bijwerkingen afnamen en het middel dus beter werd verdragen. In onze studie werd slechts bij één van de 28 deelnemende patiënten een tijdelijke afname van uitzaaiingen gezien en 7 patiënten hadden tijdelijke stabilisatie van de ziekte.

In fase I onderzoek wordt altijd de eerste ervaring met een middel bij patiënten opgedaan. Al het onderzoek dat eraan vooraf is gegaan heeft zich in het algemeen in het laboratorium of in proefdieren afgespeeld. Tijdens fase I onderzoek wordt daarom ook altijd geprobeerd om zoveel mogelijk te weten te komen over de biologische effecten bij de mens. Wij waren ten eerste geïnteresseerd in de effecten die IL-12 bij patiënten heeft op verschillende componenten van het immuunsysteem (hoofdstuk 3). De eerste IL-12 toediening gaf een verhoging van bloedspiegels van een hele reeks andere cytokines, terwijl de witte bloedcellen, die belangrijke functies vervullen bij de afweer, tijdelijk vanuit het bloed naar de organen trokken. Na de volgende IL-12 injecties bleef stijging van de meeste cytokines uit, evenals de effecten op de witte bloedcellen. Van één cytokine, namelijk IL-10, bleven de spiegels in de loop van volgende IL-12 injecties wèl stijgen. IL-10 wordt beschouwd als een remmer van het immuunsysteem, en de hoge spiegels hebben vermoedelijk de andere effecten van IL-12 onderdrukt.

In een aantal studies, verricht door andere onderzoeksgroepen, waren na IL-12 injectie ernstige bloedingen bij patiënten opgetreden. Daarom bestudeerden we de effecten van IL-12 op de stolling (hoofdstuk 4). Nu bleek dat IL-12 injectie een langdurig stimulerend effect op de oplossing van bloedstolsels heeft, hetgeen bloedingsneiging kan geven.

Bij ernstige bacteriële infecties zoals bloedvergiftiging treden er in het lichaam heftige ontstekingsreacties op, die uiteindelijk schadelijk voor de organen kunnen zijn. In deze situaties zijn IL-12 bloedspiegels vaak hoog. We bestudeerden daarom of IL-12 ontstekingseffecten tot gevolg heeft (hoofdstuk 5). IL-12 bleek, in de dosis die voor fase II onderzoek geadviseerd werd, inderdaad een stimulus voor gegeneraliseerde ontstekingseffecten te zijn. Bij lagere doseringen was dit nauwelijks het geval.

De eerste fase II studie met IL-12 is inmiddels in andere onderzoekscentra bij patiënten met uitgezaaide niercel kanker uitgevoerd. Helaas bleek IL-12 niet werkzaam. Het ontbreken van anti-tumor effecten lijkt gerelateerd aan de uitdoving van immunologische effecten die door ons werd waargenomen bij herhaalde IL-12 injectie.

Wij veronderstellen dat in het menselijk lichaam feedback mechanismen in werking treden, zoals bijvoorbeeld productie van IL-10, in antwoord op de potentieel schadelijke ontstekingseffecten die door ons werden waargenomen na relatief hoge dosering IL-12.

Recent onderzoek toont aan dat IL-12 desondanks een belangrijk immunotherapeutisch middel kan worden. Kanker vaccinatie is een andere vorm van immunotherapie waarnaar op dit moment wereldwijd veel onderzoek wordt gedaan. Vaccins tegen kanker bestaan vaak uit eiwitten afkomstig van kankercellen, waaraan een hulpstof is toegevoegd die de afweer tegen deze eiwitten moet stimuleren. Een dergelijke hulpstof heet een adjuvans. Elders uitgevoerde studies in proefdieren en patiënten hebben nu aangetoond dat IL-12 krachtige adjuvante effecten heeft. Hoofdstuk 6 geeft hiervan een overzicht. Omdat alle klassieke adjuvante stoffen lokale ontsteking veroorzaken, lijken de ontstekingseffecten van IL-12 een belangrijke rol te spelen bij de adjuvante werking. In lage doseringen blijkt IL-12 het meest werkzaam als adjuvans.

Conclusie

Bij herhaalde toediening van relatief hoge doseringen IL-12 doven de immunologische effecten van IL-12 uit. Dit is vermoedelijk de oorzaak van de teleurstellende behandelingsresultaten in fase II onderzoek. Recent hebben kanker vaccinatie studies aangetoond dat IL-12 in lage doseringen een krachtige adjuvante werking heeft. Lokale ontstekingseffecten lijken hierbij van groot belang te zijn. Echter, wij toonden aan dat hoge doseringen IL-12 tot gegeneraliseerde ontstekingseffecten kunnen leiden. Deze potentieel schadelijke

reacties activeren waarschijnlijk feedback mechanismen die de therapeutische effecten van IL-12 teniet kunnen doen.
IL-12 is een veelbelovend adjuvans voor kanker vaccinaties, en in lage dosis waarschijnlijk het meest effectief.

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