Phase I Study of Intraperitoneal Recombinant Human Interleukin 12 in Patients with Müllerian Carcinoma, Gastrointestinal Primary Malignancies, and Mesothelioma[1]

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ABSTRACT

Purpose: **The purpose is to determine dose-limiting toxicity, pharmacokinetics, pharmacodynamics, and immunobiology after i.p. injections of recombinant human IL-12 (rhIL-12).**

Experimental Design: **rhIL-12 was administered to 29 previously treated patients with peritoneal carcinomatosis from Müllerian carcinomas, gastrointestinal tract carcinomas and peritoneal mesothelioma in a Phase I trial. rhIL-12 doses were increased from 3 to 600 ng/kg. Three or more patients at each level received weekly i.p. injections of rhIL-12.**

Results: **Dose-limiting toxicity (elevated transaminase) occurred in 2 of 4 patients at the 600 ng/kg dose. More frequent toxicities included fever, fatigue, abdominal pain, nausea, and catheter-related infections. Ten patients received 300 ng/kg with acceptable frequency and severity of side effects. Two patients (one with ovarian cancer and one with mesothelioma) had no remaining disease at laparoscopy. Eight patients had stable disease and 19 progressive disease. At 300 ng/kg i.p., IL-12 was cleared from peritoneal fluid in a biphasic manner with a terminal-phase half-life of 18.7 h; peritoneal fluid levels of IL-12 5 min after i.p. injection were 100–200 pg/ml, and serum levels reached ~10 pg/ml between 24 and 36 h. IL-1-α, IL-2, IL-10, tumor necrosis factor α, and IFN-γ were determined in serum and peritoneal fluid. IFN-γ, IL-10, and tumor necrosis factor α were detected most frequently. Immunobiological effects included peritoneal tumor cell apoptosis, decreased tumor cell expression of basic fibroblast growth factor and vascular endothelial growth factor, elevated IFN-γ and IFN-inducible protein 10 transcripts in peritoneal exudate cells, and increased proportions of peritoneal $CD3^+$ relative to $CD14^+$ cells.**

Conclusions: **rhIL-12 at 300 ng/kg by weekly i.p. injection is biologically active and adequately tolerated for Phase II studies.**

INTRODUCTION

IL[3]-12 is a heterodimeric glycoprotein comprising M_r 35,000 and M_r 40,000 subunits (p35, p40) linked by a disulfide bond. Biological activity requires coexpression of the subunits. IL-12 is produced by macrophages and dendritic cells in response to various stimuli (1, 2). NK and T cells express receptors for IL-12, which activate and induce these cells (3, 4) to proliferate in culture (2, 5, 6), secrete IFN-γ (2, 7, 8), and to exhibit inhibitory activity against tumor cell lines (6, 7, 9). Murine IL-12 stimulates T-cell progenitors to mature into Th_1 cells and stimulates Th_1 cells to secrete IFN-γ (10) while inhibiting the activation of Th_2 cells (11). In humans, T-cell clones may secrete both IL-10 and IFN-γ in response to exogenous and endogenous antigens (12). A combination of rIL-12 and other IFN-γ-inducing agents leads to additive effects (5, 7, 8). rIL-12 induces tumor regression in experimental models (13, 14). T-cell infiltrates in these studies were predominantly of the $CD8^+$ phenotype, suggesting an important functional role for $CD8^+$ T cells. IL-12 produces an antiangiogenic effect that is abrogated by neutralizing antibodies to IFN-γ, possibly mediated by the CXC chemokine IP10 (15–17). Clinical responses to rhIL-12 administered by i.v. or s.c. injection have occurred in renal cell carcinoma (18), melanoma (19), and cutaneous T-cell lymphoma (20) at doses from 50 to 500 ng/kg.

Peritoneal carcinomas that metastasize from ovarian and

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[3] The abbreviations used are: IL, interleukin; NK, natural killer; MNL, mononuclear leukocyte; PEC, peritoneal exudate cell; rIL, recombinant IL; rhIL, recombinant human IL; IP10, IFN-inducible protein 10; EOC, epithelial ovarian cancer; TCR, T-cell receptor; DLT, dose-limiting toxicity; MTD, maximum-tolerated dose; TNF-α, tumor necrosis factor α; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; CT, computed tomography; CR, complete remission.

other peritoneal malignancies are associated with substantial numbers of MNLs, primarily T-cells, NK, and dendritic and B cells (21, 22). PECs from EOC patients include T cells, many of which express activation characteristics (23, 24). TCR sequencing analysis has revealed high proportions of clonally expanded $\alpha\beta$-TCR$^+$ tumor-infiltrating lymphocytes, which appear to be responding to unknown tumor antigen(s) *in vivo* (25). These findings overall suggest that tumor-infiltrating lymphocyte associated with EOC and, possibly, gastrointestinal tract tumors could be responsive to i.p. treatment with rhIL-12. Because recombinant IFN-α2b became the first recombinant cytokine to be tested in i.p. clinical therapy trials (26), the i.p. route has been used for several other cytokines [reviewed in Freedman *et al.* (24)]. In a previous study of i.p. recombinant human IFN-γ by Pujade-Lauraine *et al.* (27), significant responses were observed even in patients with either microscopic or macroscopic disease.

This is the first report of a clinical trial in which patients with peritoneal carcinomatosis attributable to ovarian and other intra-abdominal malignancies received i.p. injections of rhIL-12. Prior studies with other cytokines of lower molecular weight (24) suggest a lengthened peritoneal cavity residence times for rhIL-12. A weekly schedule of i.p. injections with slow dose escalation was considered appropriate for a Phase I clinical trial.

PATIENTS AND METHODS

Eligibility included histopathological or cytological documentation of ovarian or extraovarian Müllerian or gastrointestinal carcinoma or mesothelioma with surgical, radiological, or clinical evidence of peritoneal involvement. Except for mesothelioma, all patients had to have received adequate chemotherapy. Other criteria included no significant adhesions or signs and symptoms of bowel obstruction, prior radiation therapy limited to the pelvis, and no concurrent chemotherapy, immunotherapy, or radiotherapy. Successful placement of a peritoneal catheter and Zubrod performance status of $\leq$2 were also required. Hematological requirements included absolute granulocyte count $\geq$ 1500 cells/mm^3, platelets $\geq$ 100,000 cells/mm^3, lymphocytes $>$ 800/m^3, hemoglobin $\geq$ 9 g/dl, bilirubin $\leq$ 1.5 mg/dl, aspartate aminotransferase or alanine aminotransferase $<$ 2.5$\times$ the upper limits of normal, serum creatinine $\leq$ 1.5 mg/dl, and serum albumin $\geq$ 3.5 g/dl. Patients with histories of autoimmune disease on chronic steroid treatment or testing positive for hepatitis C or B were ineligible. Systemic chemotherapy had to be completed at least 3 weeks before entry (6 weeks after mitomycin C and 3 months after pelvic radiation). Patients were ineligible if they had intra-abdominal tumors $\geq$ 10 cm in maximum diameter, hepatic or extra-abdominal disease symptomatic or maximum diameter $\geq$ 2 cm, brain metastasis, and clinical or electrocardiogram findings indicating significant heart disease, positive pregnancy test, active ulcer, or inflammatory bowel disease.

Study Design. The study was a single-arm nonrandomized, Phase I trial, approved by the National Cancer Institute's Cancer Therapy Evaluation Program. Patients were treated at the University Clinical Research Center at the University of Texas Memorial Hermann Hospital after obtaining informed consents approved by both M. D. Anderson Cancer Center and Memorial Hermann Hospital Institutional Review Boards. A percutaneous i.p. French no. 5-7 catheter was placed in the Interventional Radiology Department at M. D. Anderson Cancer Center as described previously (24). Catheter position and adequacy of distribution was ascertained by fluoroscopy. Patients received prophylactic antibiotic treatment with ciprofloxacin, 500 mg p.o. twice a day 3 times/week, and patients allergic to ciprofloxacin received trimethoprim/sulfamethoxazole, 160/800 mg 3 times/week (24). Acetaminophen was taken as needed for fever that followed rhIL-12 injections. In the absence of ascites, $\sim$1 liter of D5W/1/4 NS was injected i.p. to facilitate peritoneal cavity distribution of rhIL-12. Additional fluid was injected as necessary to facilitate specimen collection for laboratory studies. rhIL-12 was injected weekly through the catheter, followed by a 100-ml D5W1/4 NS flush. Treatment was given on an outpatient basis. Patients were monitored for 72 h after administration of the first rhIL-12 dose and for 24 h after each subsequent dose. Four weeks of treatment constituted a cycle. Patients were evaluated for response after two cycles. All patients were monitored for toxicity.

Cohorts of 3 patients received a starting dose of 3 ng/kg. Entry to the next dosage level was permitted if at least 2 of 3 patients at the prior level had been evaluated for 2 weeks without DLTs. Ten patients were treated at the MTD to obtain a sufficient number of observations for correlative laboratory studies and to assess more reliably toxicity at that dose level. Toxicity was measured according to Common Toxicity Criteria of the National Cancer Institute. MTD was defined as the dose at which no more than 1 in 6 patients developed a DLT. DLT was defined as reversible grade 3 or 4 hematological toxicity or reversible nonhematological grade 3 toxicity (grade 2 for neurotoxicity) in 33% of patients treated at a given dose level. The following grade 3 or 4 toxicities were not considered dose limiting: grade 3 elevation of liver function tests returning to grade 1 or lower by the next scheduled dose; grade 3 anemia; grade 3 leukopenia, neutropenia, or thrombocytopenia returning to grade 1 or lower by the next scheduled dose; or grade 3 or 4 lymphopenia. MTD was assessed only on patients receiving their first courses of therapy. Three patients/cohort were entered on dose escalation, and if one of the 3 patients developed a DLT, up to an additional 3 patients were entered at that dose level. If 2 patients in a cohort developed DLT, accrual to the cohort was stopped, and the MTD was determined to have been exceeded. Dosage was then decreased to the previous dose level.

Assessment of Response. Patients were assessed for response by CT scan, peritoneal fluid cytology and physical examination at the end of each 8 weeks of rhIL-12 treatment. Patients with minimal tumor burden were evaluated by laparoscopy.

Pharmacology of i.p. rhIL-12. Serum and peritoneal concentrations of rhIL-12 were determined during the initial administration of rhIL-12 at the following time intervals: preinjection and then after 5, 10, 15, 30 min, then 1, 2, 4, 6, 8, 10, 24, 36, and 48 h. An additional blood sample was taken at 72 h. All samples were stored at -60°C and were batch processed later.

Cytokine Analysis. Analyses of different cytokines (IL-1, IL-2, IL-10, TNF-α, and IFN-γ) in serum and peritoneal fluid were performed using specific, quantitative ELISA kits (R&D Systems, Inc., Minneapolis, MN). Analysis of IL-12 was

performed using an ELISA modification of procedures and reagents supplied by Genetics Institute, Inc. (Cambridge, MA). Lowest detectable levels in pg/ml were 0–1.6 for rhIL-12, 0–3 for IFN-γ, 0–0.18 for TNF-α, 0–7 for IL-2, 0–1 for IL-10, and 0–1 for IL-1.

Quantitative Analysis of RNA Extracts for Specific Transcripts of IFN-γ, IL-10, and IP10. Quantitative analysis of cDNAs prepared from PECs for IFN-γ, IL-10, and IP10 were determined by reverse transcription-PCR. Cytokine and IP10 analyses were performed, and transcripts were quantitated as we have described previously (23, 28). The IP10 primers used in these experiments were forward 5′-GGAACCTCCAGTCTCAGCACC-3′ and reverse: 5′-CAGCCTCTGTGTGGTCCATCC-3′ and produce a 375-bp product (15).

Immunohistochemistry for Surrogate Angiogenesis Markers bFGF and VEGF. Immunohistochemical staining and quantitation of antigens on PECs were performed using methods that we have used in previous i.p. therapy trials (22). Briefly, cytospin slides were prepared from PECs, fixed with acetone, and frozen at −20°C until ready for staining (22). Primary antibodies were added for 2.5 h at room temperature. Slides were incubated for 1 h with secondary antibodies and then with avidin-biotin-peroxidase complex (1/50) for 30 min. 3,3′-Diaminobenzidine plus nickel chloride substrate was used, and slides were counterstained with 1% methyl green. Primary antibodies included anti-VEGF epitope-specific polyclonal rabbit antibody at a concentration of 1/20 (BioGenex, San Ramon, CA), anti-bFGF affinity-purified rabbit polyclonal antibody, 0.1 μg/ml (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit IgG (Vector Laboratories, Burlingame, CA), and negative control rabbit serum (Supersensitive Rabbit; BioGenex). Secondary antibodies included rabbit biotinylated antigoat IgG at a concentration of 1/200 (Vector Laboratories). Quantitation was done by measuring the mean absorbance parameter on a Samba 4000 image analyzer (22).

Terminal Deoxynucleotidyl Transferase-mediated Nick End Labeling Assay for Quantitating Apoptosis. The method developed by Dr. Mien-Chie Hung (University of Texas M. D. Anderson Cancer Center) for monitoring patients on gene therapy trials was used (29). In brief, cytospin slides were prepared from peritoneal fluids and fixed in 4% paraformaldehyde at 4°C for 24 h. Cells were washed in PBS, air dried, immersed in 0.5% triton100, diluted in PBS for 15 min, and washed in distilled water. Deproteination was done with 1:1000 proteinase K (20 μg/ml) at 37°C for 15 min. Slides were washed in distilled water and 1× TDT buffer 0.3 units/μl (1:40). Biotinylated dUTP in 1× TDT buffer was added, slides were incubated for 1 h at 37°C, washed six times in PBS, and blocked with 1 or 10% normal horse serum. Avidin-biotin-peroxidase complex 1:100 was added for 1 h. Slides were washed and developed with 0.125% 3-amino-9-ethylcarbazole buffer and counterstained with Meyer's hematoxylin.

Immunofluorescence Analysis of Peritoneal MNL Population Determination. MNLs from PECs that expressed differentiation antigens and early intermediate and late activation markers were determined by immunofluorescence using a fluorescence-activated cell sorter, as we have described previously (22).

Table 1 Patient characteristics

Total patients	29
Sex	
Male	4
Female	25
Median age (range)	50 (26–80)
Performance status (Zubrod)	
0	20
1	9
Race	
Asian	1
Hispanic	4
White	24
Primary tumor type	
Ovarian/extraovarian, Müllerian	20
Colon	4
Appendix	1
Small intestine	1
Stomach	1
Pancreas	1
Mesothelioma	1
Prior therapy	
Surgery	29
Chemotherapy	28
Immunotherapy	3
Hormone therapy	1
Radiotherapy	1
Prior chemotherapy: no. of regimens	
0	1
1	16
2	7
3	2
>3	2
No. of agents	
0	1
1	4
2	13
3	6
>3	4
Prior immunotherapy: no. of regimens	
0	27
1	1
2	1
Patients with minimal residual disease	7
Patients with bulky disease	22

RESULTS

Twenty patients with prior diagnoses of primary ovarian or extraovarian Müllerian carcinoma, 8 with gastrointestinal cancer and 1 patient with epithelial papillary mesothelioma, were enrolled between October 24, 1997, and November 27, 2000 (Table 1). All had peritoneal carcinomatosis. Of these patients, 28 had received prior chemotherapy, and the patient with peritoneal mesothelioma had received only surgical treatment. Minimal residual disease (<1 cm) was present in 7 patients.

Toxicity. No life-threatening toxicities (summarized in Table 2) were observed in this study. DLTs were encountered in 2 of 4 patients who received 600 ng/kg; grade 3 transaminase elevation was seen that did not return to grade 1 or less by the time the next dose was due, and the MTD was therefore exceeded. Dose reductions to 300 ng/kg were required for these patients. One of 11 patients evaluable for toxicity required

Table 2 Toxicity

Dose in ng/kg	3			10			30			100			300			600		
Patient no.	3			3			3			3			11			4		
Toxicity grade	All	3	4	All	3	4	All	3	4	All	3	4	All	3	4	All	3	4
Nausea				2			1			1			4	1	0	2		
Vomiting							1			1			1			1	1	0
Fatigue	1			1			3			2			6	1	0	3	2	0
Diarrhea										1			3					
Fever							1			1	1	0	9	2	0	3		
Infection	3			1	1	0							1					
Abdominal Pain	1			1			2			2			5	1	0			
Arthralgia				1			2			1			1			1		
Thrombocytopenia				1						1								
Anemia	1			3			2						1			1		
Granulocytopenia	2						2						3	1	0	2	1	0
Lymphopenia	3	2	1	1	1	0	2			1	0	1	1	1	0	1		
Transaminases	1			1			1						3			2	2	0
BUN				1														
Proteinuria	2						1											

reduction from 300 to 100 ng/kg because of delayed recovery from a grade 3 transaminase elevation. Two patients at the 100 ng/kg level received 2 and 6 unscheduled doses of 500 ng/kg, respectively, because of a dilution error. These dose variances occurred after completion of the response assessments and without any toxicities above grade 1. During the same study period, 2 patients at the 300 ng/kg level received 3 and 1 unscheduled doses of 1500 ng/kg, respectively. Except for grade 3 lymphopenia in 1 patient and grade 3 fever in the other, the following toxicities experienced by these 2 patients were lower than grade 3: flu-like symptoms; chills; headache; abdominal pain; anorexia; diarrhea; aspartate aminotransferase elevation; headache; chills; granulocytopenia; and alkaline phosphatase elevation. The fever secondary to administration of IL-12 occurred fairly regularly, ~12 h from i.p. administration and was resolved within 48 h. Subsequent patients received 300 ng/kg dose levels to characterize toxicity better and to perform laboratory correlates on a larger cohort. Five of 29 (17%) patients experienced catheter-related infections. Five of 29 patients required catheter replacements once (2) or twice (3).

Tumor Responses. Tumor responses in relation to dose level, number of courses, and tumor type are shown in Table 3. Of 9 patients treated at 3, 10, and 30 ng/kg, 8 had progressive disease and 1 stable disease. At 100 ng/kg, a patient showed complete response at laparoscopy, supported by negative cytology tests, negative peritoneal biopsies for tumor, normalization of CA-125, and resolution of CT abnormalities. Two other patients treated at this dose level had stable disease based on CA-125 levels and CT results. At the 300 ng/kg dose, a patient with epithelial papillary mesothelioma was found at surgical restaging to have only granulomas without residual mesothelioma. This patient had progressive disease after a prior abdominal surgery had demonstrated multiple sites that were positive for epithelial papillary mesothelioma. Before the treatment with i.p. rhIL-12, the patient was re-explored because of abdominal pain and a pelvic mass that involved a segment of small bowel. A segment of bowel was resected and had multiple nodules of mesothelioma. Of 29 enrolled patients, 2 experienced complete response (the patient with mesothelioma is without progression at 2 years), 8 had stable disease, and the remainder had progressive disease.

Table 3 Patient response

IP rhIL-12 (ng/kg)	Primary type (no. of patients)	Courses (1 course = 4 weeks)	Response
3	Ovary (2), colon (1)	4, 3, 2	NC[a,b], PD, PD
10	Ovary (3)	2, 2, 2	PD, PD, PD
30	Ovary (1), colon (1), appendix (1)	4, 2, 2	NC, PD, PD
100	Ovary (3)	4, 6, 3	NC, NC, CR[b]
300	Ovary (1)[c], small bowel (1)[c], ovary (1)	5, 4, 1	NC, PD, PD
	Ovary (2), extraovarian Müllerian (1),	2, 1, 1	PD, PD, PD
	Ovary (3)	3, 2, 2,	NC, PD, PD,
	Colon (1), pancreas (1), extraovarian (1)	2, 1, 2,	PD, PD, PD,
	Mesothelioma (1)	6	CR[d]
600	Stomach (1), ovary (2), extraovarian Müllerian (1)	1, 2, 4, 2	PD, PD, NC, NC

[a] NC, no change; PD, progressive disease.
[b] Peritoneal lesion at left paracolic site cleared at second look laparoscopy when deep pelvic nodal metastases were discovered and resected. Continued on study until periaortic adenopathy developed.
[c] Patients who received initial, unplanned dose of 1500 ng/kg.
[d] Complete remission at laparoscopy, normalization of CA-125, resolution of CT abnormality.
[e] Surgical reevaluation negative for tumor. Dose reduced by 50% on week 3 because of grade 3 transaminase toxicity.

Peritoneal Cytology and Ploidy Analyses. Pretreatment peritoneal specimens obtained from the 29 patients for cytology testing were positive for malignant cells in 17 patients, abnormal without presence of malignant cells in 7 patients, and normal in 5 patients. DNA ploidy analyses revealed aneuploid patterns in 14 of 17 positive specimens, in 1 of 7 abnormal specimens, and in 0 of 5 cytologically normal specimens. Conversely, diploidy was present in 3 of 17 positive specimens, in 6 of 7 reported as abnormal, and in all 5 reported as

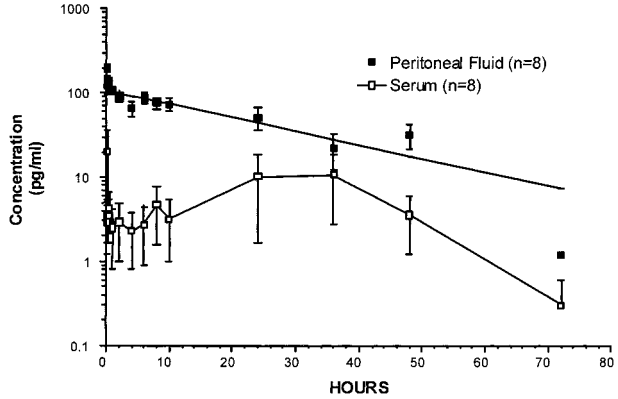

Fig. 1 Clearance of IL-12 after i.p. administration of 300 ng/kg of rhIL-12.

normal. In the EOC patient who had a CR, peritoneal cytology results returned to normal, and aneuploidy returned to a diploid pattern. In the patient with mesothelioma who had a CR, clusters of mesothelial cells with papillary features suspicious for mesothelioma were present in the peritoneal fluid upon study entry. At the time of second look, laparoscopy the peritoneal washings were negative for mesothelioma.

Pharmacokinetics of IL-12. Levels of IL-12 in serum and peritoneal fluid were monitored and assessed in most of the patients and were found to be variable. Eight patients at the 300 ng/kg dose level provided more extensive pharmacokinetic observations. Clearance of IL-12 from peritoneal fluid was subjected to nonlinear regression analysis (PK Analyst; MicroMath, Inc.) for development of pharmacokinetic parameters. Clearance of IL-12 closely fit a two-compartment mathematical model. The values for initial- and terminal-phase half-lives were 1.5 min and 18.7 h, respectively. Calculated residence time was 26.8 h and the area under the concentration curve was 2670 pg/ml × h. The appearance and clearance of IL-12 in serum could not be fitted accurately, however, maximal concentration of IL-12 occurred between 24 and 36 h after injection (Fig. 1) and was 10–50-fold lower than the contemporaneous peritoneal fluid concentrations.

Pharmacodynamics of IL-12. Serum and peritoneal fluid samples obtained at the time of the pharmacokinetic analysis were assayed for IFN-γ, TNF-α, IL-2, IL-10, and IL-1α by quantitative ELISA. These cytokines were selected as indicators of activation of T cells (IFN-γ, TNF-α, IL2, IL-10), NK cells, (IFN-γ), or macrophages (TNF-α, IL-Iα, IL-10) and because of the functions of these cytokines. Because cytokine production was not detected in patients treated at rhIL-12 doses < 30 ng/kg, results shown are for patients treated at doses of ≥ 30 ng/kg (Table 4, *a* and *b*). Peritoneal fluid concentrations of IFN-γ increased after i.p. rhIL-12 injection in 13 of 19 (68%) patients tested. At the 300 ng dose level, IFN-γ was detected in 8 of 9 peritoneal fluids and 9 of 10 serum specimens taken after treatment. Similar patterns of cytokine production were detected in contemporaneous serum specimens. Levels of IFN-γ started to increase at 6 h and generally achieved peak concentrations ~24 h after i.p. IL-12 administration (Fig. 2*A*). The median times to maximum cytokine concentration in the peritoneal fluid were: IFN-γ, 36 h (range, 10–48); TNF-α, 36 h (range, 24–48); IL-10, 30 h (range, 6–72); and IL-1α, 30 h (range, 24–36). The median times to maximum cytokine concentration in serum were: IFNγ, 36 h (range, 24–48); TNF-α, 36 h (range, 6–48); and IL-10, 24 h (range, 6–48). Several patients had constitutive levels of IL-10 or TNF-α in peritoneal fluid and in serum. At the 300 ng dose level, no significant difference was detected between the pre- and posttreatment values for IL-10 (Fig. 2*B*).

Expression of IFN-γ/IL-10 and IP10 Transcripts in cDNAs from PECs before and after Treatment. IFN-γ RNA transcripts from the pretreatment PECs ranged from 0 to 1260 agm/μl with a median level of 0 agm ($n = 19$). After one to three i.p. injections of rhIL-12, IFN-γ transcripts ranged from 0 to 2,697,400 agm, with a median level of 1,000 agm ($n = 19$). IL-10 pretreatment transcripts ranged from 0 to 19,680 agm, with a median value of 0.6 agm ($n = 19$), and posttreatment IL-10 transcripts ranged from 0 to 19,760 agm, with a median value of 0 agm. The CXC chemokine IP10 transcript pretreatment ranged from <0.134 to 60,200 agm, with a median value of 1,880 agm/μl ($n = 19$). Post-i.p. rhIL-12, IP10 transcript levels ranged from 0.135 to 1,035,400 agm, with a median value of 1,809 agm/μl ($n = 19$).

The effects of i.p. rhIL-12 injections on the expression of transcripts for IFN-γ and IP10 were examined after one and two injections for all patients for whom follow-up values were available and for ovarian and gastrointestinal cancer patients separately. Using the signed rank test of change in IFN-γ from the pretreatment, significant differences for treatment effect were detected for IFN-γ/transcripts after two i.p. injections ($P = 0.016$) for both ovarian and gastrointestinal patients ($n = 14$). There was also a significant difference for the ovarian cancer patients ($P = 0.031$) but not for the smaller group of patients with gastrointestinal tract cancers ($P = 1.0$).

Overall, no significant differences were seen in the transcripts for IP10 or IL-10; however, the patient with EOC who had pathologically confirmed complete response to i.p. rhIL-12 showed a decrease in IP10 transcripts that paralleled an increase in IFN-γ transcripts and a decrease in IL-10 transcripts and their encoded proteins (Tables 5 and 6).

Modulation of bFGF and VEGF Expression on PECs after i.p. rhIL-12 Injections. Expression of surrogate markers of angiogenesis, VEGF and bFGF, were measured by quantitative immunohistochemical analyses using the mean absorbance (see Table 7). After weekly i.p. injections of rhL-12, patients had significant decreases in bFGF expression after either one or three injections. Differences in expression of VEGF at the same time points were not significant, but the responding patient whose cytokine data was shown in Tables 5 and 6 showed a decrease in bFGF expression from 10.8 (pretreatment) to 1 (1 week posttreatment) and 5.9 (4 weeks posttreatment) and VEGF of 3.5, 0.7, and 1.1 at the same time points.

Apoptosis of Tumor Cells and Peritoneal MNL during i.p. rhIL-12. PECs that include tumor cells, MNLs, and other cells were monitored for apoptosis in tumor cells and leukocytes in 4 patients at either 300 or 600 ng/kg doses. An increase in the proportions of apoptotic tumor cells was detected in 3 of 4 patients tested after i.p. injections of rhIL-12. Lower proportions

Table 4a Endogenous cytokine concentrations in peritoneal fluids pre- and post-i.p.[a] injection of first rhIL-12 dose

IP rhIL-12 Dose (ng/kg)		IFN-γ No. of positive/total	IFN-γ (pg/ml)[a] Median (range)	TNF-α No. of positive/total	TNF-α (pg/ml) Median (range)	IL-2 No. of positive/total	IL-10 No. of positive/total	IL-10 (pg/ml) Median (range)	IL-1α No. of positive/total	IL-1α (pg/ml) Median (range)
30	Pre	0/2	0 (0–0)[b]	1/2	0.78 (0–1.56)	0/1	0/1	0 (0–0)	0/1	0 (0–0)
	Post	2/2	304.87 (53.45–556.28)	1/2	14.83 (0–29.65)	0/1	0/1	0 (0–0)	0/2	0 (0–0)
100	Pre	0/3	0 (0–0)	1/3	0.003 (0–0.06)	0/2	0/2	0 (0–0)	0/3	0 (0–0)
	Post	1/3	0 (0–117.15)	2/3	19.16 (0–20.85)	0/2	1/3	0 (0–47.71)	0/3	0 (0–0)
300	Pre	1/10	0 (0–10.12)	1/9	5.13 (0–10.25)	0/8	3/9	0 (0–21.24)	0/3	0 (0–0)
	Post	8/9	278.10 (0–472.10)	3/8	0 (0–17.69)	0/8	5/10	5.01 (0–44.62)	1/3	0 (0–9.11)
>300	Pre	0/5	0 (0–0)	0/6	0 (0–0)	0/5	1/6	0 (0–17.34)	1/6	0 (0–10.89)
	Post	3/5	121.72 (0–497.88)	1/4	7.26 (0–14.51)	0/5	2/6	0 (0–66.98)	1/6	0 (0–68.93)
No. of positive/total	Pre	1/20		3/20		0/16	4/18		1/13	
No. of positive/total	Post	14/19		7/17		0/16	8/20		2/14	

[a] Posttreatment concentrations = maximum concentrations reached >24–48 h.
[b] Zero values = below lower limits of detection.

Table 4b Endogenous cytokine concentrations in serum pre- and post-i.p.[a] injection of first rhIL-12 dose

IP rhIL-12 Dose (ng/kg)		IFN-γ No. of positive/total	IFN-γ (pg/ml)[a] Median (range)	TNF-α No. of positive/total	TNF-α (pg/ml) Median (range)	IL-2 No. of positive/total	IL-10 No. of positive/total	IL-10 (pg/ml) Median (range)	IL-1α No. of positive/total	IL-1α (pg/ml) Median (range)
30	Pre	0/2	0 (0–0)[b]	1/2	1.16 (0–2.32)	N/A	0/1	0 (0–0)	0/1	0 (0–0)
	Post	1/1	40.41	1/2	6.78 (0–13.55)	0/1	0/1	0 (0–0)	0/2	0 (0–0)
100	Pre	0/3	0 (0–0)	2/3	10.55 (0–23.04)	0/2	0/3	0 (0–0)	0/3	0 (0–0)
	Post	1/3	68.46	2/3	22.53 (0–32.23)	0/2	1/3	6.52 (0–13.03)	0/3	0 (0–0)
300	Pre	1/10	0.73 (0–1.45)	0/8	0 (0–0)	0/8	2/9	0 (0–18.32)	0/3	0 (0–0)
	Post	9/10	137.92 (0–521.25)	2/8	0 (0–48.95)	0/8	5/9	2.49 (0–31.98)	0/3	0 (0–0)
>300	Pre	1/5	2.92 (0–5.84)	1/6	3.23 (0–6.45)	0/5	0/6	0 (0–0)	0/6	0 (0–0)
	Post	3/5	11.74 (0–398.21)	1/4	0 (0–20.23)	0/5	5/6	35.81 (0–50.91)	0/6	0 (0–0)
No. of positive/total	Pre	2/20		4/19		0/15	2/19		0/13	
No. of positive/total	Post	14/19		6/17		0/16	11/19		0/14	

[a] Posttreatment concentrations = maximum concentrations reached >24–48 h.
[b] Zero values = below lower limits of detection.

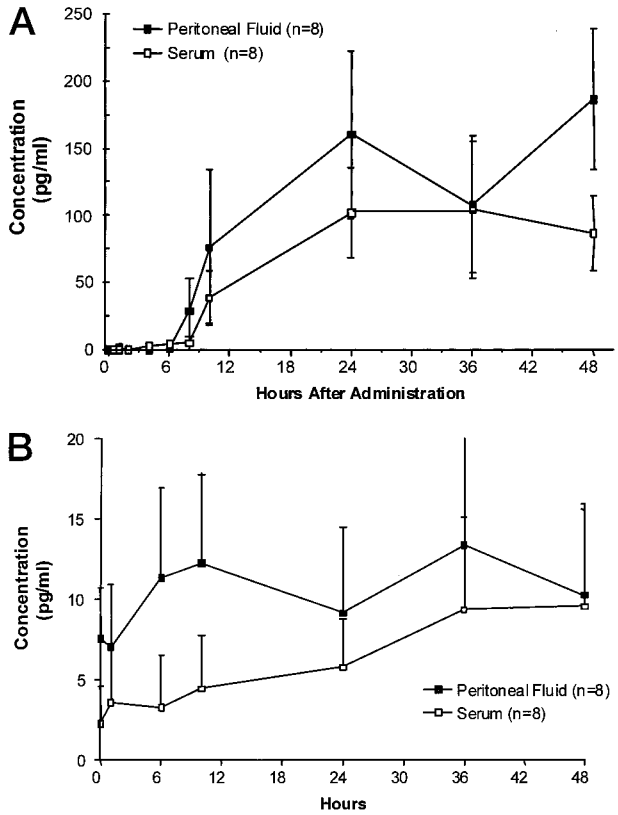

Fig. 2 *A*, peritoneal fluid and serum concentrations of IFN-γ after i.p. administration of 300 ng/kg rhIL-12. *B*, peritoneal fluid and serum concentrations of IL-10 after i.p. administration of 300 ng/kg rhIL-12.

Table 5 Cytokine and chemokine responses during treatment of a responding EOC patient

Quantitative measurement of cytokine/chemokine transcripts in RNA extracts of PECs.

Cytokine/ Chemokine	Pretreatment (agm/liter)	Posttreatment (agm/liter)	
		Week 1	Week 3
IFN-γ	<2,470	38,660	15,970
IL-2	0	0	0
IL-10	3,190	830	500
IP10	520	620	810

of apoptotic MNLs were also detected in some of these patients (Fig. 3). None of the patients who had apoptosis studies done were clinical responders.

Modulation of Peritoneal MNL Populations after i.p. rhIL-12. PECs obtained before and 1 week after treatment were examined for the proportion of MNLs that express the CD14 ($n = 14$) and CD3 ($n = 9$) lineage markers. Eleven of 14 specimens analyzed were from patients above the 10 ng dose. The mean (±SE) proportion of cells that were positive for the CD14 antigen was 52.1 ± 6.1% pretreatment and 23.8 ± 4.7% post-i.p. rhIL-12. The mean (±SE) proportion of cells that were positive for CD3 antigen was 44.4 ± 8.6% pretreatment and 63.5 ± 7.2% posttreatment. According to the matched-pairs *t* test, there was a significant increase in $CD3^+$ cells ($P = 0.032$) relative to the decrease in $CD14^+$ cells ($P = 0.001$). PECs were also examined for changes in early intermediate and late activation markers, including the CD25, CD56, CD45RO, and $CD4^+$ and $CD8^+$ subsets that expressed HLA-DR, CD69, and CD45RO, respectively. Changes in the proportions of activation markers were variable and were not significant statistically (data not shown). By the signed rank test $CD8^+RA^-RO^+$ cells showed an increasing trend at 1 week posttreatment ($P = 0.068$). Mean pretreatment values were 12.5 ± 1.9% and posttreatment were 17.1 ± 2.3%.

Table 6 Cytokine and chemokine responses during treatment of a responding EOC patient

Maximum cytokine levels and timing in peritoneal fluid (PF) and serum (S) following i.p. injection of rhIL-12

Cytokine	IL-12		IFN-γ		TNF-α		IL-2		IL-1α		IL-10	
Source	PF	S	PF	S	PF	S	PF	S	PF	S	PF	S
Max pg/μl	22	12	117	69	19	23	0	0	0	0	0	0
Time (hrs)	48	10	36	48	36	6						

DISCUSSION

i.p. administration of rhIL12 on a weekly outpatient schedule is feasible and has limited immediate or cumulative toxicity effects to doses of 300 ng/kg. Clinical activity and IL-12 levels were detected above the 30 ng dose level, and a majority of patients produced increased concentrations of IFN-γ protein or cytokine-specific transcripts for IFN-γ after treatment. IFN-γ production was also detected in the serum of these patients but at lower levels than in the peritoneal cavity. The presence of higher peritoneal concentrations of IL-12 and its induced-cytokine IFN-γ relative to their serum concentrations could contribute to an overall lower intensity of systemic IFN-γ-related toxicities. One of 2 patients who received unscheduled doses of 1500 ng/kg did not develop toxicities above grade 2, except for lymphopenia. Interestingly, IL-1 but not IFN-γ was detected in this patient. The patient had received high-dose chemotherapy and stem cell transplantation 6 months earlier. Perhaps this patient failed to produce IFN-γ because of a dysfunctional lymphocyte response, which has been observed after high-dose chemotherapy (30).

TNF-α was detected in 7 of 17 patients after treatment, and increased concentrations of both IFN-γ and TNF-α were detected in the 2 patients who responded. IFN-γ and TNF-α have synergistic cytotoxic antitumor activity in preclinical studies (31). An antiangiogenic mechanism has also been suggested for IFN-γ and TNF-α through down-regulation of αVβ-3 integrin receptor activity, resulting in apoptosis of the tumor neovasculature (32). bFGF expression on PECs decreased after treatment with i.p. IL-12, but expression of VEGF was not significantly decreased overall. However, substantial decrease in VEGF expression was observed in the responding EOC patient. The decreased expression of bFGF and VEGF and increased expression of IP10 transcript in certain patients suggest the possibility of different antiangiogenesis mechanisms in these patients.

Lymphocyte populations responsible for producing IFN-γ

Table 7 Modulation of FGF_2 and VEGF expression on peritoneal carcinoma cells after one or three cycles of i.p. rhIL-12

	Mean optical density measurements[a]				
Antigen	Pretreatment (*N*)[b] Mean ± SE	PO1 injection (*N*) Mean ± SE	PO3 injection (*N*) Mean ± SE	(I) (II)	Pre *versus* PO1 Pre *versus* PO3
FGF_2	7.17 ± 1.15 (11)	4.30 ± 0.53 (10)	4.19 ± 0.89 (9)	(I)	$P = 0.0045$
				(II)	$P = 0.0066$
VEGF	3.73 ± 0.74 (10)	3.59 ± 0.85 (9)	2.90 ± 0.48 (9)	(I)	ns
				(II)	ns

[a] Calculated values after subtraction of isotype controls.
[b] *N* = number of patients; ns = not significant; PO1: postinjection 1; PO3: postinjection 3.

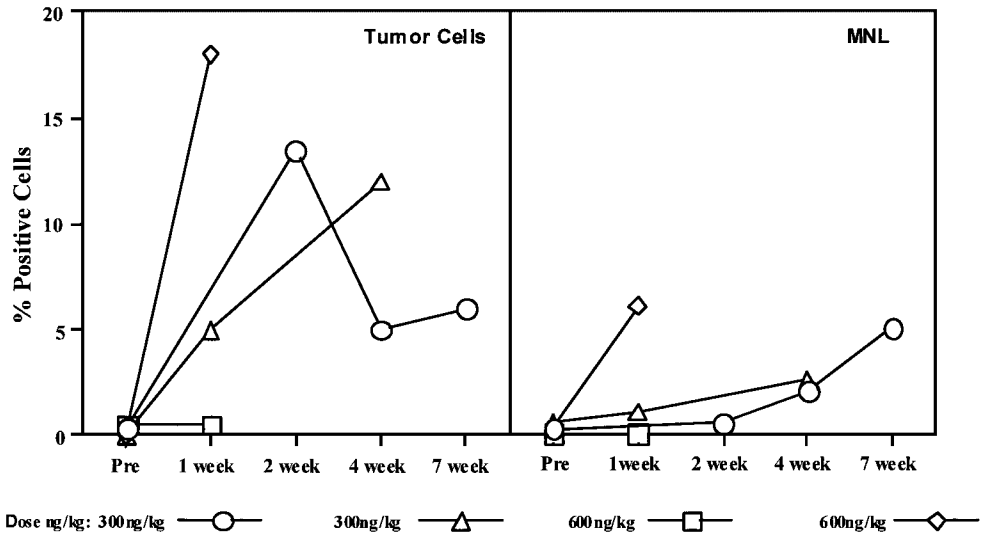

Fig. 3 Proportions of tumor cells and MNLs showing apoptosis in terminal deoxynucleotidyl transferase-mediated nick end labeling assay after i.p. injection of rhIL-12.

after i.p. IL-12 have not been determined. IL-2 secretion was not detected either pre- or posttreatment. Although IL2 is transiently expressed by producer lymphocytes, *in vitro* experiments have shown that peak IL-2 gene expression occurs at 6 h with peak protein production at ~12–24 h (33). In consideration of evidence that the pharmacokinetic characteristics of the peritoneal cavity favor a lengthened residence time for molecules the size of cytokines, it would not be unexpected if IL2 were detected after i.p. injections of rhIL12. IL-2 could possibly be produced by PECs at autocrine levels, however, no increase in IL-2 transcripts was detected in these patients. It is also possible that IFN-γ production in the absence of IL-2 could indicate an NK-type response or an associated T-cell defect. Decreased expression of TCR phosphorylation and of CD3 ζ chain expression has been observed in PECs and solid tumors of certain patients, however, this effect was reversible after *in vitro* treatment of these cells with rhIL-2 (34, 35). Lack of IL-2 production by T cells could also be attributable to absent costimulation or presence of suppressor cell populations that inhibit T-cell activation directly or through production of molecules such as IL-10 and transforming growth factor β. Peritoneal macrophages with the $CD14^+DR^-IL\text{-}10^+$transforming growth factor $\beta 2^+$ phenotype have been identified from EOC patients (36). Although 13 of 19 patients produced IFN-γ after injection of i.p. rIL-12 and 8 of 20 patients produced IL-10, significant increases in IL-10 were not detected at the 300 ng dose. In contrast, we have observed a positive relationship between i.p. rIL-2 administration and IL-10 production *in vivo* (22, 37). It may be that an adaptive immune response (tumor vaccine) in combination with the IL-12 could be an additional approach that warrants investigation in ovarian cancer. Such an approach has been studied in patients with melanoma who received a multipeptide vaccine combined with low-dose IL-12 (38). If specific T-cell responses are deficient or disrupted *in vivo*, it may be possible to overcome this problem by providing costimulation or by developing strategies to interfere with suppressor cell or inhibitory cytokine pathways.

We conclude that IL-12 at a weekly dose of 300 ng/kg by i.p. injection has acceptable safety and immunobiological effects that provide a basis for a Phase II trial in patients with small residual disease, recently initiated in EOC patients who have completed first-line chemotherapy.

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