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Recruitment of dendritic cells and enhanced antigen-specific immune reactivity in cancer patients treated with hr-GM-CSF (Molgramostim) and hr-IL-2: results from a phase Ib clinical trial

P. Correale [a], G. Campoccia [b], K.Y. Tsang [c], L. Micheli [d], M.G. Cusi [e], M. Sabatino [a], G. Bruni [d], S. Sestini [e], R. Petrioli [a], D. Pozzessere [a], S. Marsili [a], G. Fanetti [b], G. Giorgi [d], G. Francini [a,*]

[a] *Division of Medical Oncology, University of Siena, Viale Bracci 11, 53100 Siena, Italy*

[b] *Blood Bank, University of Siena, Viale Bracci 11, 53100 Siena, Italy*

[c] *Experimental Oncology Section, Laboratory of Tumor Immunology and Biology National Cancer Institute, National Institute of Health, 80100 Bethesda, MD, USA*

[d] *Department of Pharmacology, "Giorgio Segre", University of Siena, Viale Bracci 11, 53100 Siena, Italy*

[e] *Department of Molecular Biology, University of Siena, Viale Bracci 11, 53100 Siena, Italy*

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Abstract

Experimental findings suggest that granulocyte-monocyte-colony stimulating factor (GM-CSF) synergistically interacts with interleukin-2 (IL-2) in generating an efficient antigen-specific immune response. We evaluated the toxicity, antitumour activity and immunobiological effects of human recombinant (hr)-GM-CSF and hr-IL-2 in 25 cancer patients who subcutaneously (s.c.) received hr-GM-CSF 150 μg/day for 5 days, followed by hrIL-2 s.c. for 10 days and 15 days rest. Two of the most common side-effects were bone pain and fever. Of the 24 patients evaluable for response, 3 achieved partial remission, 13 experienced stable disease, and 8 progressed. Cytokine treatment increased the number of monocytes, dendritic cells (DC), and lymphocytes (memory T cells) in the peripheral blood and enhanced the antigen-specific immunoreactivity of these patients. Our results show that the hr-GM-CSF and hr-IL-2 combination is active and well tolerated. Its biological activity may support tumour associated antigen (TAA)-specific anticancer immunotherapy by increasing antigen presenting cell (APC) activity and T cell immune competence *in vivo*. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Phase Ib clinical trial; Immunotherapy; hr-GM-CSF; hr-IL-2; Dendritic cells; Antigen-specific immunoreactivity

1. Introduction

The antitumour activity of subcutaneous (s.c.) low-dose human recombinant interleukin-2 (hr-IL-2), administered alone or in combination with other cytokines, has been demonstrated in patients with renal cell carcinoma [1,2]. Interest in the chronic subcutaneous (s.c.) administration of hr-IL-2 has increased as a result of various biological observations concerning the modulation of specific cytotoxic T lymphocytes (CTL), $CD4^+$ T cell populations and cytokine profiles in cancer patients [3]. On the basis of the results of numerous trials, cancer immunotherapy with hr-IL-2 is currently used in Europe in an outpatient setting and has practically no toxicity [4].

It is widely accepted that a T lymphocyte response to tumour associated antigens (TAA) is essential for obtaining efficient and prolonged tumour rejection *in vivo* [1,2]. T lymphocytes recognise tumour and viral protein antigens as small peptides (the products of intracellular degradation) bound to MHC molecules on target cells [5]. Tumour cells express major histocompatibility complex (MHC)-bound tumour antigen peptides and can thus be recognised and killed by activated cytotoxic T lymphocytes, but they cannot start a demonstrable CTL immune reaction since they lack the

* Corresponding author. Tel.: +39-577-586-231-369429; fax +39-577-586139.

E-mail address: francini@unisi.it (G. Francini).

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expression of costimulatory molecules such as B.1 (CD80) and B7.2 (CD86), which are indispensable for the activation of CTL precursors [5]. However, these molecules are expressed by professional antigen presenting cells (APC) such as dendritic cells (DCs), which play a central role in initiating an antigen-specific immune reaction as a result of their ability to uptake, process and present the antigen proteins released by the tumour or virally-infected cells [6,7].

DCs have been extensively studied over the last 5 years, and various authors have described protocols for their *in vitro* generation from human peripheral blood mononuclear cells (PBMCs) or bone marrow stimulated with granulocyte-macrophage monocyte-colony stimulating factor (GM-CSF) combined with IL-4 or tumour necrosis factor (TNF)-α, respectively [8,9]. These protocols have made it possible to develop new immunotherapeutic strategies using DCs as a living immune-adjuvant for therapeutic anticancer vaccines [9].

hr-GM-CSF is the key agent in the promotion of both *in vitro* and *in vivo* DC proliferation and differentiation [10], and also regulates the proliferation and differentiation of granulocyte, eosinophil, and macrophage progenitors [11,12]. The clinical antitumour activity of hrGM-CSF (Molgramostim) has been tested in patients with renal cell carcinoma, and been found to have less antitumour activity than hr-GM-CSF and an acceptable degree of toxicity [13,14]. More recent *in vitro* studies have shown that hr-GM-CSF interacts with hrIL-2 to generate human prostate-specific antigen PSA-specific CTL lines [15].

On the basis of these observations, we performed a clinical trial in which s.c. hr-GM-CSF and s.c. hr-IL-2 were sequentially administered to patients with advanced cancer in order to verify the hypothesis that hr-GM-CSF promotes the proliferation and differentiation of APCs such as DCs, monocytes and macrophages, which may initiate a tumour-specific immune reaction that is subsequently sustained and amplified by hr-IL-2.

The primary aim of the study was to evaluate the toxicity profile and biomodulatory potential of this new treatment schedule; the secondary aim was to make a preliminary investigation of its antitumour activity.

2. Patients and methods

2.1. Patient eligibility

Between August 1998 and July 2000, we enrolled 25 patients (14 females and 11 males) with a median age of 69.5 years (range 58–75 years) and histologically confirmed diagnoses of renal cell carcinoma (11), colorectal carcinoma (8), soft tissue sarcoma (2), non small-cell lung carcinoma (1), pancreas carcinoma (1), prostate carcinoma (1) and malignant melanoma (1). All of the patients had to have advanced disease, an Eastern Cooperative Oncology Group (ECOG) performance status of $\leqslant 2$, a life expectancy of >3 months, normal renal and hepatic function, a white blood cell (WBC) count of >2500 cells/μl, haemoglobin >90 g/L, platelets $>100\,000$ cells/μl, a cardiac ejection fraction of $>26\%$, and a normal electrocardiogram. The exclusion criteria were valvular and wall motion abnormalities, central nervous system (CNS) involvement, second tumours, liver or renal abnormalities, active infectious disease, or a history of cardiovascular disease. This study was approved by the local (University) Ethics Committee, and conducted according to the applicable good clinical practice (GCP) guidelines. All of the patients gave their written informed consent.

2.2. Treatment schedule

The patients received 150 μg of hr-GM-CSF (Schering-Plough, Corp.) s.c. for 5 days (days 1–5) and then hr-IL-2 (Chiron) s.c. twice a day for 10 days (days 6–15); this was followed by 15 days rest (days 16–30) before a second cycle was begun. If the side-effects were negligible (grade <3) after two cycles, the hr-IL-2 dose was increased. The daily dose was escalated from 0.25 to 3.0 MIU ($M=\times 10^6$ in) the same patient and in consecutive cohorts of 3 patients every two cycles (every 2 months) according to Fibonacci's schedule [16]. The 15-day rest period between cycles was adopted in order to avoid the negative affects on effective lymphocyte activity that may be caused by the long-term continuous administration of hr-IL-2 [5,17], as well as to enhance tolerability and reduce treatment costs. The final group of 3 patients (nos. 23–25) enrolled at the end of the study were administered what appeared to be the most effective hr-IL-2 dose, and were evaluated only in terms of clinical response and toxicity.

2.3. Clinical assessments

A complete history was taken of all of the patients before they started treatment, and they also underwent a physical examination, complete blood counts, serum chemistry and an echographic examination of left ventricular anatomy and function. Complete disease staging was performed at baseline and after three and six cycles of treatment by means of chest X-ray, computed tomography (CT) of the chest and abdomen, and echography of the liver and pelvis. Full blood counts, biochemistry profile, liver function tests, electrocardiogram (ECG) and urinalysis were performed weekly. All of the eligible patients were evaluated for toxicity according to standard World Health Organization (WHO) criteria [18]. The patients who completed at least three treatment cycles were evaluable for response. If the patients responded or had stable disease, the treatment was

continued until the occurrence of disease progression or unacceptable toxicity.

2.4. Response criteria

A complete response was defined as the complete disappearance of all known measurable disease for at least 1 month, and a partial response as a $\geqslant$50% decrease in known lesions lasting for at least 1 month. The area of two-dimensional lesions was defined as the product of the longest diameter multiplied by the greatest perpendicular diameter; disease stabilisation was defined as a $<$50% decrease or $\leqslant$25% increase in evaluable lesions lasting for 1 month without the appearance of new lesions, and progressive disease as a $>$25% increase in known disease or the appearance of new lesions [18].

2.5. Selection of patients undergoing immunological testing

The first 18 patients enrolled in the study were divided into six cohorts of 3 individuals receiving different doses of IL-2 and evaluated immunologically. The results of conventional immunological testing were normal as defined by a normally delayed cutaneous hypersensitivity to *mumps*, *Candida* and *Trichophyton* antigens, and normal lymphocyte proliferation to concavalin A (Con A). These patients did not show any evidence of immunodepression as defined by the following criteria: non-reactive HIV testing; no other diagnosis of altered immune function, including eczema, atopic dermatitis or autoimmune disease (neutropenia, thrombocytopenia, haemolytic anaemia, systemic lupus erythematosus, Sjogren's syndrome, sclerodermia, myasthenia gravis, Goodpasture's syndrome; Addison's disease, Hashimoto's thyroditis, active Graves' disease); no concurrent steroid use; no other serious intercurrent illness. All of the immunological experiments were performed in the same three patients belonging to a specific hr-IL-2 cohort throughout the study.

2.6. Peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from heparinised blood samples (drawn on days 1, 5 and 15 of each cycle) by means of Ficoll Hypaque lymphocyte separation medium (ICN, Costa Mesa, CA, USA), immediately frozen in fetal bovine serum (FBS) (Gibco) with 10% dimethyl sulphoxide (DMSO), and stored at -80°C.

2.7. Antigen-specific proliferation

The PBMCs were seeded in 96-well plates (Falcon Corp.) at a final dilution of 4×10^4 cells per well in a volume of 100 μl. One hundred microlitres of fresh complete medium Roswell Park Memorial Institute (RPMI)-1640 with 10% FBS, 2 mM L-glutamine, antibiotics and 1 mM N2-hydroxyethyl piperazine-N′2-ethanesulphonic acid (HEPES) containing no reagent, 10 μg of influenza virosomes or 50 μg phytohaemoagglutinin (PHA) were subsequently added to the wells in triplicate. The PBMCs were pulsed with [^{3}H]-thymidine (1 μCi per well, Amersham; 1 mCi = 37 kBq) after 3 days in culture and harvested 18 h later using a Packard Micromate cell harvester. DNA thymidine incorporation was measured as counts per minute (cpm) by means of liquid scintillation counting using a Packard Matrix 96 β counter. The results from triplicate wells were averaged as mean cpm $\pm$ standard error of the mean (SEM), with the values observed in the cells cultured in medium alone being considered a negative control. The results were expressed in the form of a stimulation index (SI) (the cpm of PBMCs exposed to influenza virosomes/the cpm of PBMCs cultured in medium alone).

2.8. Serum cytokine determination

The levels of TNFα, interferon gamma (IFNγ), IL-10, IL-5, and IL-4 were determined in serum samples collected on days 1, 5 and 15 of each cycle. The sera were stored at -80°C and thawed immediately before processing. The determinations were performed in triplicate using solid phase sandwich enzyme linked-immunosorbent assay (ELISA) kits all purchased from EuroClone Ltd, UK with the exception of the IL-5 kit that was purchased from Bio-source International Inc, CA, USA. The spectrophotometric readings were made using a multiwell reader (Biorad). The amount of cytokine in each sample was determined by extrapolating the optical density (OD) values to cytokine concentrations using the standard curve, and quantitated using Biorad Microplate Manager IV software. The minimum detectable concentrations were 10 pg/ml for TNFα, 5 pg/ml for IFNγ, 5.85 pg/ml for IL-5, 12.5 pg/ml for IL-10 and 0.5 pg/ml for IL-4.

2.9. Immunocytometric analysis

The PBMCs were thawed 24 h before the assay, incubated overnight at 37°C in 5% CO_2 in complete (RPMI-1640, GIBCO) medium containing 10% FBS, 2 mM L-glutamine, antibiotics and 1 mM HEPES. The cells were washed three times with cold Ca^{++} and Mg^{++} free Dulbecco's phosphate buffer saline (DPBS) and stained for 1 h with fluorescent-labelled monoclonal antibodies (MAbs). They were then washed a further three times with cold DPBS, resuspended in DPBS at a concentration of 1×10^6 cells/ml, and analysed using a Becton Dickinson FACScan equipped with a blue laser with an excitation of 15 nW at 488 nm. The data were gathered from 10 000 stored live cells, and used to generate the results [19]. The following mouse MAbs were used: anti-CD4 fluorescein (FITC), anti-CD8 phycoerythrin con-

jugate (PE); anti-CD3 FITC, anti-CD56 PE, anti-CD19 PE, anti-CD45Ro PE, anti-CD16 FITC, anti-CD11c PE, anti-human leucocyte antigen Dr (HLA-Dr) FITC, anti-CD80 PE, anti-HLA-Dr FITC, anti-HLA-I A/B/C PE, anti-CD11c FITC, anti-CD83 PE, anti-CD80 FITC, anti-CD86 PE, anti-CD34 FITC, anti-CD14 PE, anti-HLA-A,B,C FITC, anti-IgG1 FITC and anti-IgG2a PE (isotype controls). All of the MAbs were purchased from Becton Dickinson with the exception of anti-CD34 (purchased from Orthon Medica Corp).

2.10. Statistical considerations

The differences between the mean values of three determinations from three different experiments (±S.D.) were calculated using Student's two-tailed *t*-test for paired samples, and the differences between the patient cohorts receiving different hr-IL-2 doses were calculated using Student's two-tailed *t*-test for unpaired samples. A *P* value of <0.05 was considered statistically significant. The data were analysed using Stat View statistical software (Abacus Concepts, Berkeley, CA, USA) [20].

3. Results

3.1. Treatment response

19 of the 25 patients entering the study had received at least one line of previous treatment. A final 3 patients (nos. 23–25) enrolled in July 2000 were administered what appeared to be the most biologically and clinically active dose of hr-IL-2 (1 MIU/day), and were evaluated only for clinical response and toxicity.

One patient with prostate cancer voluntarily decided to discontinue the treatment at the end of the first cycle and was lost to follow-up, and 2 patients stopped the therapy because of a rapid worsening in their general conditions due to progressive disease.

There were three major clinical responses (partial remissions) (one colon and two renal cell carcinomas) and 13 cases of disease stabilisation lasting more than 3 months. 15 patients received a clinical benefit in terms of the quality of life and improved symptoms as evaluated by means of psychometric tests [16] and the ECOG scale [18,21] (Table 1).

Table 1
Patient characteristics

P	PS	TT	Disease sites	Previous treatment	TTP (months)	R
1	1	CRC	Liver; nodes lung	5-FU/FA, CPT-11, TOM	5	SD
2	0	PSC	Bone	Goserelin, DOX; F, A	ND	ND
3	0	CRC	Liver; nodes pelvis	5-FU/FA; CPT-11, TOM	5	SD
4	2	CRC	Liver; lung	5-FU CI; l-MIT.C	5	SD
5	2	RCC	Liver	VBL + IFN-α, hr-IL-2	3	PD
6	2	CRC	Liver; lung	5-FU/FA	3	PD
7	2	CRC	Liver; lung; bone	5-FU/FA,CPT-11, l-MIT.C	1	PD
8	1	CRC	Liver	5-FU/FA; l-MIT.C	9	PR
9	2	STS	Soft tissue	Radiotherapy; ADIC	9	SD
10	1	NSCLC	Liver	VP-16, CDDP, hypertermia	3	PD
11	1	CRC	Liver, peritoneum	5-FU/FA, TOM	NA	PD
12	1	CRC	Liver	5-FU/FA; DOX	3	PD
13	2	RCC	Nodes pelvis, lung	IFN/VBL, MAP; hr-IL-2	9	SD
14	1	RCC	Lung, nodes	IFN/VBL, hr-IL-2	9	SD
15	2	RCC	Bone	IFN/VBL	6	SD
16	1	RCC	Lung, nodes	IFN/VBL, hr-IL-2	6	SD
17	2	MM	Liver	Radiotherapy	NA	PD
18	1	STS	Soft tissue	None	6	SD
19	2	PAC	Nodes, liver, lung	5-FU/FA/gemcitabine	3	PD
20	0	RCC	Bone	Surgery	9+	PR
21	1	RCC	Kidney, nodes	Surgery	5+	PR
22	1	RCC	Kidney, nodes	Surgery	3+	SD
23	1	RCC	Kidneys	None	3+	SD
24	0	RCC	Nodes	Surgery	3+	SD
25	0	RCC	Lung, nodes, bone	Surgery, 5-FA/FU + hr-IL-2, s.c. hr-IL-2	3+	SD

PS, performance status; TTP, time to progression; SD, stable disease; PR, partial remission; PD, progression of disease; IFN, recombinant alpha2 interferon; 5-FU, 5-fluorouracil; FA, folinic acid; TOM, raltitrexed (tomudex); VBL, vinblastine; l-MIT.C, mitomycin C; DOX, doxorubicin; CRC, colorectal carcinoma; RCC, renal cell carcinoma; MM, malignant melanoma; STS, soft-tissue sarcoma; PSC, prostate carcinoma; PAC, pancreas adenocarcinoma; NSCLC, non-small cell lung cancer; hr-IL-2, human recombinant interleukin-2; F, Flutamide; A, cyproterone acetate (Androcur); CI, continuous infusion; MAP, medroxy progesterone acetate; ADIC, polychemotherapy with doxorubicin and dacarbazone; NA, not applicable; ND, not determined.

Table 2
Systemic side-effects

Side-effects	Dose of hr-IL-2 0.25	0.5	1.0	1.7	2.5	3.0
Anorexia	0	0	0	1[a]	1[a]	2
Cutaneous induration	2[a]	3[a]	2[a]	2[a]	2[a]	2[a]
Bone pain	2[a]	2[a]	2[a]	2[a]	2[a]	1[a]
Fever	2[a]	3[a]	1[a]	2[a]	2[a]	3[a]
BUN increase	0	0	0	0	0	0
Creatinine increase	0	0	0	0	0	0
Thrombocytopenia	0	0	0	1[a]	1[a]	0
Thrombocytosis	1[a]	0	0	1[a]	0	0
Hypotension	0	0	1[a]	1[a]	0	0
Nausea/vomiting	1[a]	1[a]	2[a]	1[a]	2[b]	0
Oliguria	0	0	0	0	0	0
Headache	0	1[a]	1[a]	0	1[a]	1[a]
Fatigue	0	0	0	0	0	1[a]
Anaemia	1[a]	0	0	0	1[b]	0
Transaminase elevation	0	0	0	0	0	0
Coagulopathy	0	0	0	0	0	0
CVA	0	0	0	0	0	0

WHO, World Health Organization; BUN, blood urea nitrogen; CVA, cerebrovascular accidents.

[a] WHO grade 1.

[b] WHO grade 2.

3.2. Treatment toxicity

The treatment schedule was well tolerated, with no patient experiencing any grade 3–4 toxicity. There were no cases of leak syndrome, cardiovascular or neurological complications or infectious diseases. 2 patients experienced thrombocytopenia, 2 moderate anaemia, and 2 moderate thrombocytosis. Fever and bone pain (especially during hr-GM-CSF administration) were two of the most common side-effects, being recorded in many of the patients; however, they were mild and easily controlled by oral medication. Other side-effects were cutaneous induration, erythema and pruritis at the site of the cytokine injection. Episodes of nausea/vomiting were also recorded in a few cases at the higher hr-IL-2 doses, but these were also easily controlled by oral medication. Moderate asthenia and malaise was observed in 6 patients. None of the patients experienced any alterations in coagulation parameters, coagulopathy or cerebrovascular accidents during the treatment (Table 2).

3.3. Cytokine study

There was a significant increase in serum IFN-γ ($P=0.001$) and TNF-α ($P=0.001$) after treatment for most dose levels (Table 3), but no changes in IL-4 and IL-10 levels at any of the hr-IL-2 doses. IL-10 levels in particular, were always inferior to the minimal detectable amount. An increase in IL-5 levels was detectable in a few patients after treatment, but no statistical significance was found for day 15 versus day 1 readings: $P>0.05$ (Table 3).

3.4. Haemocytometric cell counts

An increase in the number of monocytes was detected in all of the patients on day 5 after hr-GM-CSF, with a return to baseline levels at the end of the same cycle (day 15) (Fig. 1). The number of eosinophils increased in the patients receiving higher hr-IL-2 doses (Fig. 2), and there was an increase in the number of lymphocytes after treatment at a dose of 0.5 MIU in particular (Fig. 3).

3.5. Immunocytometric analysis

3.5.1. Lymphocytes

Immunophenotypic study of PBMCs gated as lymphocytes with a high forward- and side-scatter (Fig. 4) revealed a posttreatment increase in T cells ($CD3^+$, $CD56^-$, $CD19^-$) and NK markers ($CD3^-$, $CD56^+$, $CD16^+$) (Table 4). The CD4/CD8 T cell ratio improved in almost all of the patients after the first cycle (Table 5). The increase in the number of $CD4^+$ T cells was paralleled by an increase in the expression of HLA-Dr

Table 3
Cytokine profile[z]

[hr-IL-2], MIU/day	IL-4 Day 1	IL-4 Day 15	IL-10 Day 1	IL-10 Day 15	IFNγ Day 1	IFNγ Day 15	TNFα Day 1	TNFα Day 15	IL-5 Day 1	IL-5 Day 15
0.25	0.8 (0.63)	0.7 (0.19)	< 12.5	< 12.5	15.0 (0.9)	38.6 (2.23)[a]	26.1 (5.20)	59.0 (15.1)[a]	35.2 (14.9)	35.0 (6.26)
0.5	1.5 (0.52)	1.7 (0.51)	< 12.5	< 12.5	15.5 (0.5)	32.6 (2.68)[a]	29.5 (5.36)	86.5 (18.75)[a]	18.3 (9.70)	20.39 (6.20)
1.0	1.2 (0.28)	3.0 (1.03)	< 12.5	< 12.5	18.4 (0.7)	43.5 (13.5)[a]	27.1 (5.53)	53.3 (28.74)[a]	20.3 (22.3)	28.2 (19.82)
1.7	1.1 (0.15)	2.4 (2.20)	< 12.5	< 12.5	16.0 (0.5)	26.0 (7.88)[a]	26.1 (5.80)	35.9 (4.18)	7.16 (4.1	12.9 (7.85)
2.5	1.3 (0.30)	1.1 (0.46)	< 12.5	< 12.5	17.6 (0.8)	48.3 (20.1)[a]	26.5 (4.44)	48.4 (9.28)[a]	9.93 (9.12)	21.67 (13.23)
3.0	2.3 (1.54)	1.7 (1.21)	< 12.5	< 12.5	25.7 (12.4)	49.5 (7.32)[a]	27.8 (8.34)	48.4 (13.56)[a]	8.01 (9.21)	7.95 (7.20)

Data derived from 3 different patients of each group receiving different hr-IL-2 dosages. Results are expressed as pg/ml. Numbers in parentheses represent the standard deviations (S.D.).

[a] Differences (treatment day 1 versus day 15) considered statistically significant $P<0.05$.

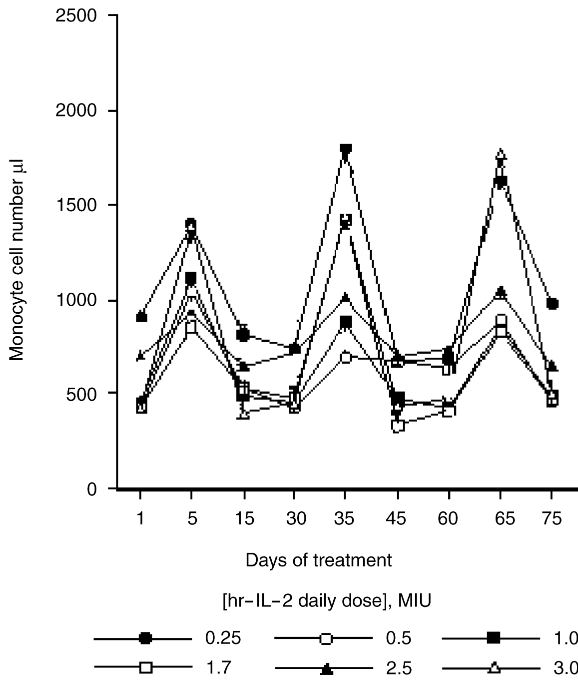

Fig. 1. Cytokine treatment increases the number of monocytes in cancer patients. Results of haemocytometric cell counts of blood samples taken from 3 different patients in each group receiving different hr-IL-2 doses. The standard deviations (S.D.) are omitted for the sake of clarity.

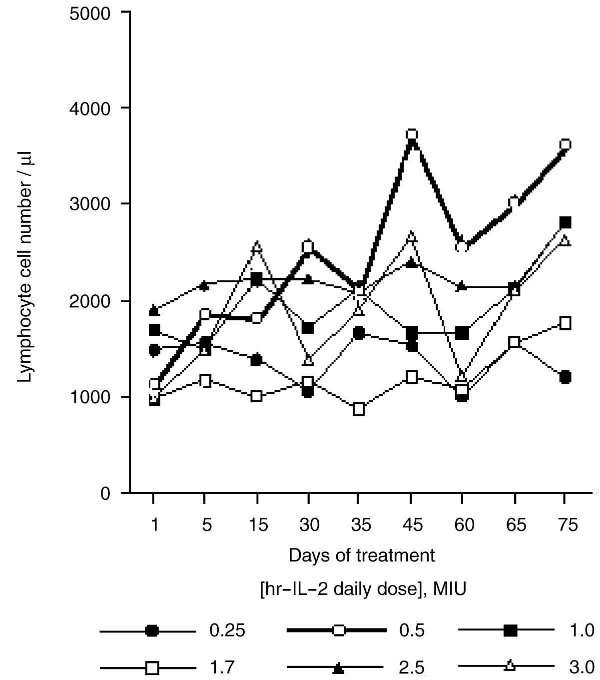

Fig. 3. Cytokine treatment increases the number of lymphocytes in cancer patients. Results of haemocytometric cell counts of blood samples taken from 3 different patients in each group receiving different hr-IL-2 doses. The standard deviations (S.D.) are omitted for the sake of clarity.

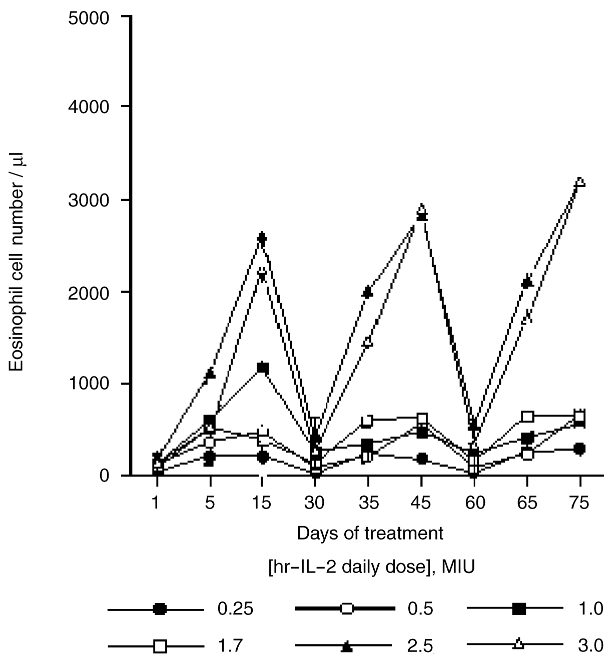

Fig. 2. Cytokine treatment increases the number of eosinophils in cancer patients. Results of haemocytometric cell counts of blood samples taken from 3 different patients in each group receiving different hr-IL-2 doses. The standard deviations (S.D.) are omitted for the sake of clarity.

($CD4^+$/HLA-Dr^+) and CD45Ro ($CD4^+$/$CD45Ro^+$) for most dose groups (Table 4), which suggests that hr-GM-CSF/hr-IL-2 immunotherapy affects the expansion of $CD4^+$ memory T lymphocytes.

3.5.2. Dendritic cells

PBMCs gated as DCs with a high forward- and side-scatter (Fig. 4) and expressing bone marrow-derived DC surface markers ($CD34^+$, $CD14^-$, $CD11c^+$, $CD86^+$, HLA-I^+, HLA-Dr^+) [8,9,22] were detected at very low levels before cytokine treatment (Fig. 5), but these significantly increased after one cycle (day 15 versus day 1: $P=0.05$; day 15 versus day 5: $P=0.001$; day 1 versus day 5: $P=0.001$) (Fig. 5 and Table 6). The most significant increase in DC markers was observed in the patients receiving 0.5 MIU of hr-IL-2 (Fig. 5). After one treatment cycle, these cells also showed a relative increase in CD83, CD80 (B7.1) (day 1 versus day 5: $P=0.063$; day 5 versus day 15: $P=0.167$; versus day 1 day 15: $P=0.014$). This last finding suggests that cytokine treatment increases the recruitment of bone marrow-derived DCs and promotes their differentiation, as suggested by the finding of an enhanced expression of CD83 [22] and costimulatory molecules such as CD80 (B7.1) and CD86 (B7.2), which are molecular structures strictly involved in antigen presentation.

3.6. Antigen-specific proliferation

Patient immune response to influenza virus particles (influenza virosomes) was tested by *means of antigen-specific proliferation* assays, which revealed an enhanced ability of PBMCs to present influenza virus antigens to antigen-specific CTL precursors *in vitro* after one treatment cycle *in vitro*.

An increased proliferative response (measured as an increased [^{3}H]-thymidine incorporation) to influenza antigens was in fact, detected in PBMC derived from patients who had received one treatment cycle (Table 7); virus particle-unexposed PBMC isolated on treatment days 1 and 15 were used as negative controls.

4. Discussion

The clinical results of the trial suggest that the treatment was well tolerated, and indicate that it has promising anti-tumour activity, as shown by the three partial remissions and 13 disease stabilisations observed in patients with very advanced disease. In comparison with other studies of hr-GM-CSF or hr-IL-2 alone or in

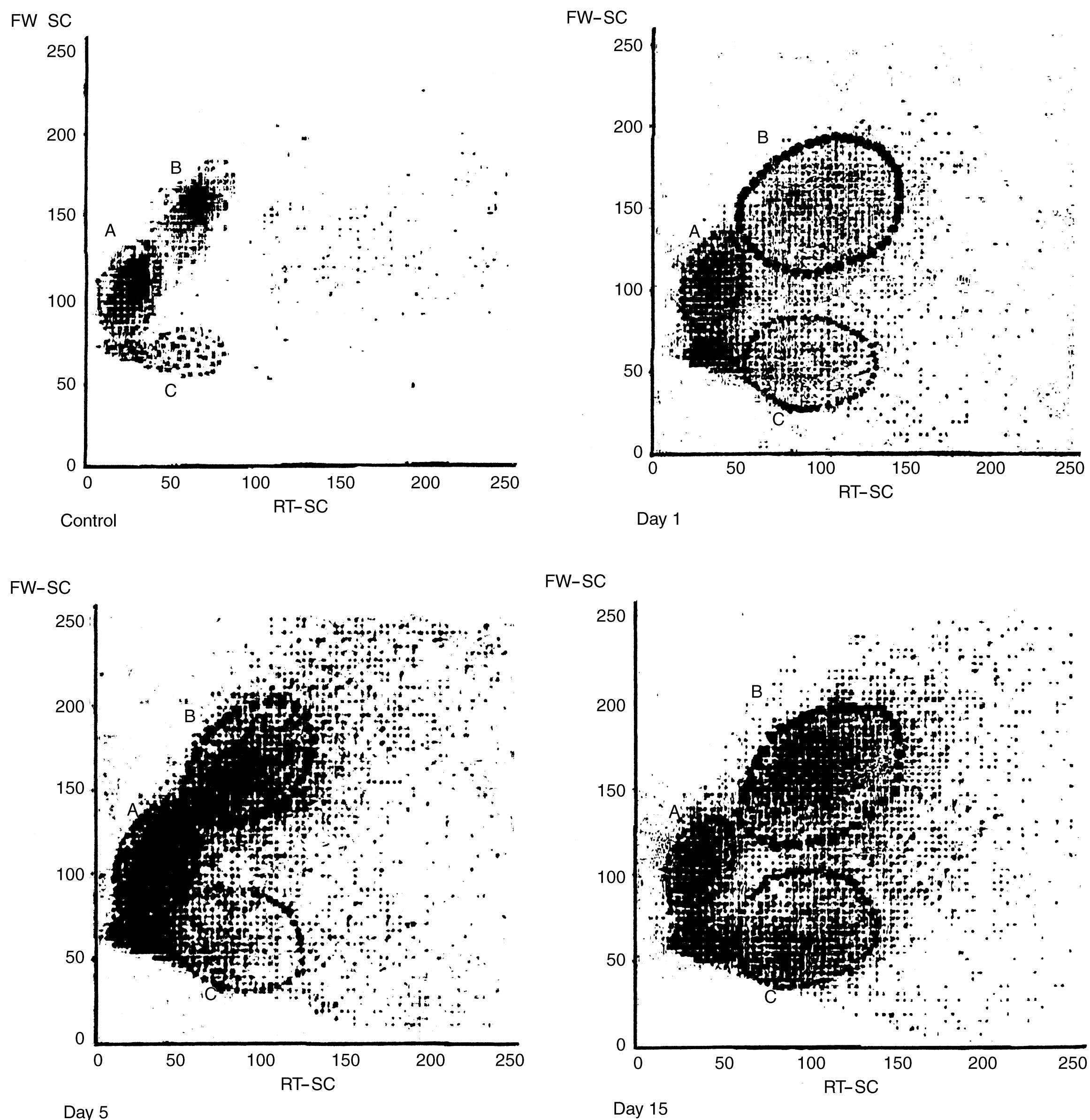

Fig. 4. Immunocytometric analysis of PBMCs: the patient PBMCs were gated with a high forward- and side-scatter. The cells in gate A are lymphocytes and those in gate B are monocytes; the cells in gate C, which were CD34$^+$/CD11c$^+$, were considered to be DCs. PBMCs from a healthy donor: gate A = 60.2%, B = 5.8%, C = 0.5%. Patient PBMCs from treatment day 1: A = 49.3%, B = 6.3%, C = 0.9%; day 5: A = 56.3%, B = 11.2%, C = 1.1%; day 15: A = 60.1%, B = 9.1%, C = 20.9%.

Table 4
Flow cytometric analysis of surface markers on PBMCs gated as lymphocytes[a]

	Lymphocyte cell surface markers					
	CD3			CD16		
hr-IL-2 dose (MIU/day)	Day 1	Day 5	Day 15	Day 1	Day 5	Day 15
0.25	56.3 (1.2)	68.7 (2.1)	78.9 (2.5)	0.9 (2.0)	12.2 (4.3)[b]	22.2 (3.5)[c]
0.5	65.3 (15.2)	69.3 (15.2)	62.3 (22.3)	1.9 (9.5)	30.1 (10.2)[b]	25.4 (11.5)[c]
1.0	77.2 (1.8)	77.5 (1.5)	85.3 (3.0)	25.2 (5.2)	20.1 (2.6)	35.2 (1.9)[c]
1.7	68.3 (13.5)	62.3 (11.5)	55.6 (22.6)	27.1 (13.4)	34.2 (7.9)	42.5 (17.2)[c]
2.5	77.2 (2.7)	79.2 (3.6)	82.6 (1.2)	17.2 (6.3)	17.3 (6.9)	25.3 (17.1)[c]
3.0	82.3 (2.3)	62.2 (10.6)	81.0 (2.0)	3.6 (2.0)	31.0 (12.3)[b]	22.2 (8.3)[c]
	$CD4^+/CD45Ro^+$			HLA-Dr		
	Day 1	Day 5	Day 15	Day 1	Day 5	Day 15
0.25	45.3 (1.2)	43.2 (1.1)	40.3 (2.5)	5.0 (1.2)	15.2 (1.8)[b]	21.0 (2.2)[c]
0.5	39.6 (5.8)	49.3 (5.2)	58.9 (9.3)[c]	20.1 (12.1)	17.5 (2.9)	23.4 (8.5)
1.0	58.5 (9.8)	65.5 (9.5)	73.8 (8.7)[c]	24.5 (12.3)	19.8 (7.5)	20.2 (10.1)
1.7	29.4 (2.5)	52.7 (8.1)[b]	54.5 (7.6)[c]	12.2 (0.5)	13.2 (1.5)	24.3 (1.3)[c]
2.5	28.6 (5.7)	29.2 (7.6)	40.5 (2.2)[c]	11.0 (7.2)	17.6 (5.6)	24.1 (5.1)[c]
3.0	49.2 (0.3)	44.6 (0.6)	59.8 (2.2)[c]	18.9 (9.8)	29.8 (2.6)[b]	25.2 (6.6)[c]

PBMC, periplural blood mononuclear cells.
[a] Data derived from three different patients in each group receiving different hr-IL-2 doses. The results are expressed as the percentage of fluorescent cells in each MAb-reactive cell sample (standard deviations (S.D.) are in parentheses): 2–4% of cells are routinely stained when treated with no priming MAbs or with an isotype-related control MAb, and so marker expression was considered negative when less than 4%.
[b] Statistically significant differences between days 1 and 5 ($P<0.05$).
[c] Statistically significant differences between days 1 and 15 ($P<0.05$).

Table 5
$CD4^+/CD8^+$ T cell ratio[a]

hr-IL-2 (MIU/day)	Day 1		Day 15	
	No. of $CD4^+$/No. of $CD8^+$	CD4+/CD8+ T cell ratio	No. of CD4+/No. of CD8+	$CD4^+/CD8^+$ T cell ratio
0.25	471 (4.72)/574 (68.88)	0.82	333 (59.94)/395 (39.5)	0.84
0.5	334 (3.35)/534 (16.2)	0.63	997 (12.00)/816 (81.6)	1.22[b]
1.0	515 (61.8)/635 (133.35)	0.81	663 (6.63)/559 (83.85)	1.19[b]
1.7	478 (76.48)/420 (50.4)	1.14	685 (68.5)/570 (57.08)	1.20b
2.5	423 (21.15)/346 (34.6)	1.22	688 (27.52)/344 (35.96)	2.00[b]
3.0	800 (32.00)/691 (27.64)	1.16	1299 (103.92)/967 (87.03)	1.34[b]

[a] Data derived from three different patients in each group receiving different hr-IL-2 doses. The results are expressed as the absolute number of $CD4^+$ and $CD8^+$ cells (standard deviations (S.D.) are in parentheses), and the $CD4^+/CD8^+$ T cell ratio. Lymphocyte cell marker expression was investigated in $CD3^+$. PBMCs gated as lymphocytes with a high forward- and side-scatter. The results are expressed as the percentage of fluorescent cells in each MAb-reactive cell sample (S.D. are in parentheses): 2–4% cells are routinely stained when treated with no priming MAbs or with an isotype-related control MAb, and so marker expression was considered negative when less than 4%.
[b] Statistically significant differences between days 1 and 15 ($P<0.05$).

combination, the side-effects recorded during the present study were much less frequent and severe [2,3,13,14,23–26]. Four studies have recently reported the side-effects and antitumour activity of combined hr-GM-CSF and hr-IL-2 treatment in advanced cancer patients. Three of these studies used s.c. hr-GM-CSF (at doses ranging from 2.5 to 10 μg/kg/day for 12–14 days) administered simultaneously or sequentially with an intravenous (i.v.) infusion of hr-IL-2 (at doses ranging from 1.5×10^6 to 11×10^6 IU, 4 days per week for 4 weeks), and mainly found it to be poorly effective with only occasional observations of minimal responses. They also highlighted the occurrence of numerous and significant side-effects, including neurological toxicity and coagulopathy [23,25,26]. The differences in the results of these studies in comparison with our own may be due to differences in the way the studies were designed and conducted, insofar as we used very low s.c. doses of hr-IL-2, and hr-GM-CSF was administered for a shorter time (5 days/30 days) at a dose of 150 μg/day. We chose to give hr-IL-2 s.c. because this modality is considered active and less toxic than i.v. infusions or boluses [4]. Other significant differences in the administration schedules could also explain the high degree of

Table 6
Flow cytometric analysis of surface markers on PBMCs gated as dendritic cells[a]

	Dendritic cell surface markers								
	CD11c			HLA-Dr			HLA-I-A,B,C		
IL-2 (MIU/day)	Day 1	Day 5	Day 15	Day 1	Day 5	Day 15	Day 1	Day 5	Day 15
0.25	68.1 (2.1)	85.2 (2.1)[b]	87.4 (2.2)*	70.9 (5.0)	86.4 (15.0)	85.1 (5.2)	79.9 (5.2)	92.1 (0.2)	89.1 (0.2)
0.5	69.3 (1.8)	97.8 (0.9)[b]	85.4 (3.4)*	81.1 (10.5)	89.8 (3.2)	79.8 (7.1)	89.3 (3.7)	91.0 (3.8)	85.9 (0.17)
1.0	65.0 (10.1)	85.2 (2.9)[b]	84.3 (2.9)*	69.8 (0.8)	84.3 (12.9)	79.3 (2.5)	83.6 (15.8)	84.3 (2.5)	87.1 (0.9)
1.7	65.4 (10.1)	59.2 (9.9)	71.5 (8.2)	68.0 (20.8)	64.1 (24.2)	82.6 (11.2)	81.2 (2.7)	90.5 (4.9)	79.1 (0.33)
2.5	62.1 (10.2)	66.3 (9.8)	70.2 (11.9)	43.9 (36.8)	42.8 (25.4)	44.9 (22.0	77.6 (7.2)	74.9 (3.5)	83.5 (1.7)
3.0	62.0 (1.0)	60.3 (0.9)	50.9 (1.9)	46.4 (38.9)	69.0 (42.8)	63.2 (37.8)	88.7 (3.8)	88.1 (0.8)	84.1 (1.6)
	CD80			CD83			CD86		
	Day 1	Day 5	Day 15	Day 1	Day 5	Day 15	Day 1	Day 5	Day 15
0.25	2.0 (1.0)	12.0 (0.9)[b]	14.0 (1.9)[c]	16.1 (2.2)	17.0 (1.2)	27.3 (2.4)[c]	57.9 (5.2)	59.9 (1.2)	84.7 (12.2)
0.5	4.1 (3.3)	11.2 (1.4)	19.2 (6.7)[c]	20.1 (13.7)	28.2 (13.8)	40.3 (11.7)[c]	61.7 (3.7)	71.6 (3.8)	95.5 (11.7)[c]
1.0	5.4 (2.7)	5.2 (0.2)	19.1 (4.8)[c]	19.3 (5.3)	19.6 (1.5)	29.2 (3.9)[c]	58.9 (15.3)	57.9 (18.5)	74.2 (13.9)[c]
1.7	7.6 (2.8)	14.4 (7.5)[b]	16.6 (2.3)[c]	20.2 (4.7)	30.5 (4.5)	42.9 (3.3)[c]	42.1 (14.7)	45.5 (4.5)	60.1 (5.3)[c]
2.5	0.9 (0.7)	4.0 (2.5)	16.1 (5.2)[c]	25.6 (2.2)	34.3 (3.4)	38.6 (2.7)[c]	51.2 (7.2)	52.0 (7.4)	70.2 (12.7)[c]
3.0	1.1 (1.1)	4.3 (1.4)	16.6 (3.0)[c]	23.7 (1.7)	25.1 (2.8)	42.3 (1.8)[c]	28.9 (3.7)	36.6 (3.8)	52.3 (11.8)[c]

[a] Data derived from three different patients in each group receiving different hr-IL-2 doses. Dendritic cell marker expression was investigated in PBMCs gated as dendritic cells with a high forward- and side-scatter: the gated cells were $CD34^+$ (>90%), $CD11c^+$ and $CD14^-$. The results are expressed as the percentage of fluorescent cells in each MAb-reactive cell sample (standard deviations (S.D.) are in parentheses): 2–4% cells are routinely stained when treated with no priming MAbs or with an isotype-related control MAb, and so marker expression was considered negative when less than 4%.

[b] Statistically significant differences between days 1 and 15 ($P<0.05$).

[c] Statistically significant differences (S.D.) between days 1 and 15 ($P<0.05$).

toxicity reported by these authors, as is suggested by the results of another phase I clinical trial reported by de Gast and colleagues, who investigated the effects of hr-GM-CSF 2 μg/kg and low-dose hr-IL-2 (2–4 MIU/m^2) given simultaneously by the s.c. route in combination with αIFN (5 MIU) for 12 days to 11 patients with renal cell carcinoma and 7 with melanoma. The toxicity of this protocol was significant, with the occurrence of one fatal cerebrovascular accident, grade 4 fever and hypotension, and grade 3 fatigue/malaise and fluid retention [24], but the clinical and biological results were very

Table 7
Antigen-specific proliferation[a]

hr-IL-2 (MIU/day)	PBMCs Day 1	PBMCs Day 15	T value
0.25	1.5 (0.52)	1.56 (0.08)	2.01[b]
0.5	0.99 (0.55)	1.97 (0.34)	2.625[b]
1.0	1.03 (0.033)	11.17 (0.14)	122.1 ($P<0.001$)
1.7	1.07(0.66)	6.11 (4.18)	2.06[b]
2.5	3.21 (0.5)	6.23 (0.9)	5.081 ($P<0.01$)
3.0	3.1 (0.65)	6.15 (0.14)	7.95 ($P<0.001$)

[a] Data derived from three different patients in each group receiving different hr-IL-2 doses. The results are expressed in the form of a stimulation index (SI): the values counts per minute (cpm) observed in PBMCs exposed to influenza virusomes/the values (cpm) observed in PBMCs cultured in medium alone. The latter values were used as a negative control. The standard deviations (S.D.) are given in parentheses. The background [^{3}H]-thymidine incorporation of the non-stimulated cells was similar under all experimental conditions, and averaged 2500–3000 cpm.

[b] Non-significant differences.

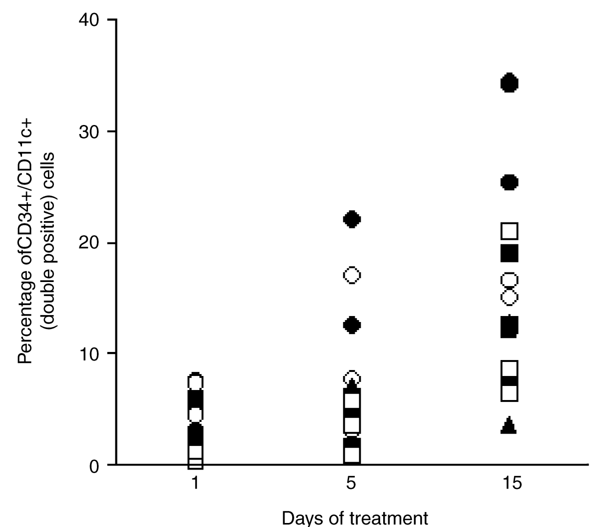

Fig. 5. Cytokine treatment expands $CD34^+/CD11c^+$ cells in patients' peripheral blood mononuclear cells (PBMCs) gated with a high forward and side-scatter as dentritic cells (DC). The results are expressed as the percentage of fluorescent cells measured by means of immunocytofluorimetric analysis. Symbols represent the percentage of DC detected at the hr-IL-2 daily dose of (△) 0.25, (●) 0.5, (○) 1, (▲) 1.7, (■) 2.5, (□)3.0 MIU. Marker expression was considered negative when lower than 4%.

similar to ours with objective responses and disease stabilisation being observed in 4 and 10 patients, respectively, and they also observed a significant increase in monocytes, eosinophils, lymphocytes, NK and activated $CD4^+$ and $CD8^+$ T cells after treatment. The lower degree of toxicity found in our study may be due to the different administration schedule of the two cytokines, to the lower amount of hr-IL-2 or to the absence of αIFN in our study protocol. It is also conceivable that our 15-day rest period between cycles enhanced the tolerability of the treatment without affecting its biological or antitumour activity, and it may have also helped in the negative feedback on antitumour effectors which can potentially be produced by the long-term continuous administration of hr-IL-2 [5,17].

Our results suggest that, with the exception of the increase in the number of eosinophils, the clinical and biological effects do not depend on the hr-IL-2 dose, as they were also evident at very low doses. This is not surprising because hr-IL-2 under different conditions induces completely divergent effects and a number of clinical trials have reported modulated CTL activity, CD4 T cell populations and cytokine profiles in cancer patients treated with very low s.c. doses [3]. We chose the sequential rather than the simultaneous use of the two cytokines because it is known that hr-GM-CSF enhances the effects of hr-IL-2 on activated lymphocytes and NK cells, and it could be expected that their simultaneous administration would be synergistic in terms of side-effects. Furthermore, it has been reported that hr-IL-2 itself induces the PBMC production of hr-GM-CSF *in vivo* and *in vitro*, and so simultaneous administration may not be justified [27]. Our study was not designed to investigate the non-specific effects of the two cytokines on whole lymphocyte, NK or lymphocyte activated killer (LAK) cell populations, but to investigate whether hr-GM-CSF enhances the number and function of APCs in cancer patients by preparing PBMCs and possibly circulating DCs to receive, process and present protein antigens released by the cancer cells to the antigen-specific CTL precursors. The subsequent administration of low-dose hr-IL-2 was specifically intended to induce the proliferation of these CTL precursors activated by antigen-loaded APCs.

Our biological results show that the cytokine sequential combination does affect the immune competence of cancer patients, a finding that may have a direct clinical application because vaccination strategies designed to activate a therapeutic immune response against tumour associated antigens (TAA) in cancer patients [28,29] are currently being investigated throughout the world [29,30]. We found that hr-GM-CSF/hr-IL-2 administration increases the percentage of PBMCs expressing bone marrow-derived DC markers and also promotes DC maturation, as suggested by the finding of a higher expression of CD83, CD80 and CD86 in PBMC populations gated as DCs. A functional *in vitro* assay also revealed a more efficient influenza virus antigen-specific proliferative response in the PBMCs of patients completing one treatment cycle, which suggests that cytokine administration enhances the ability of the APC contained in PBMCs to uptake, process and present protein antigens released by influenza virusome-infected PBMCs. The antigen particles making up the influenza virusomes could not induce any CTL proliferative response unless they were previously processed by professional APCs such as DCs or monocytes. Whether or not a similar event also occurs in response to other weaker antigens, such as those produced by cancer cells or those administered as cancer vaccine immunotherapy, will be expressly investigated in a clinical trial of tumour-associated antigen-directed vaccine therapy that has been designed to evaluate the antigen-specific CTL response in patients receiving hr-GM-CSF/hr-IL-2 or no adjuvant cytokine treatment.

Nevertheless, the present observation that hr-GM-CSF/hr-IL-2 treatment also causes a significant increase in the number of lymphocytes and $CD4^+$ memory T cells is consistent with the hypothesis that DCs play a major role in the generation of an efficient immune response. In this context, it is known that a larger number of lymphocytes and a higher $CD4^+/CD8^+$ T cell ratio are two clinical markers that positively correlate with cancer patient survival and immune competence [31]. Our data also suggest that $CD4^+$ T cells have a T helper (Th)-1 cytotoxic phenotype after treatment, because we found a posttreatment increase in serum TNF-α and IFN-γ but no change in IL-4, IL-5 and IL-10 levels. This finding may be of interest because a number of reports have suggested that, unlike Th-2 (IL-4 and IL-10 producers), Th-1 $CD4^+$ T cells promote antigen-specific cytotoxic T cell responses with anti-tumour activity [32]. Taken together, these biological results could also explain the observed clinical benefits.

In conclusion, the clinical and toxicological results observed in this study have encouraged us to continue our investigation with a phase II clinical trial in patients with renal and colon carcinoma. The immunobiological results also provide a rationale for designing a clinical trial aimed at studying the effects of hr-GM-CSF/hr-IL-2 in combination with active TAA-specific immunotherapy.

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