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Original Paper

Granulocyte-macrophage Colony-stimulating Factor Improves Immunological Parameters in Patients with Refractory Solid Tumours Receiving Second-line Chemotherapy: Correlation with Clinical Responses

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In this report, we studied the immunorestorative properties of subcutaneously administered granulocyte-macrophage colony-stimulating factor (GM-CSF) in patients with refractory solid tumours receiving second-line chemotherapy. Such patients exhibit abnormal immune responses *in vivo* and *in vitro* and, therefore, it was of interest to examine the effect of GM-CSF-induced immunomodulation on clinical response. We examined patients with primary malignant carcinomas (head and neck, $n = 10$; urogenital tract, $n = 17$; penis $n = 6$; colorectal, $n = 8$) who were treated with carboplatin (JM8), 300 ng/m^2 on days 1 and 22, leucovorin (LV), 200 mg/m^2 plus 5-fluoracil (5-FU), 500 mg/m^2 on days 8, 15 and 29 and four cycles of daily injections with placebo or GM-CSF, 300 μg/day on days 3–6, 10–13, 17–20 and 24–27. Peripheral blood was collected from the patients one day after the end of each of the four-cycle injections with placebo or GM-CSF, namely on days 7, 14, 21 and 28. Peripheral blood mononuclear cells (PBMC) were tested in the autologous mixed lymphocyte reaction (AMLR) and for natural killer (NK) or lymphokine-activated killer (LAK) cell activity. Cytokine levels in serum were measured by immunoenzymatic (ELISA) assay. A total of 21 patients received a four-cycle regimen with GM-CSF (Group 1) and 20 were similarly treated with placebo (Group 2). All received standard chemotherapy as outlined above. Before GM-CSF treatment, all patients exhibited increased serum levels of interleukin-1 (IL-1β), tumour necrosis factor-α (TNF-α), IL-6 and prostaglandin E_2 (PGE_2) and decreased serum levels of IL-2. Cellular immune responses (AMLR, NK- and LAK-cytotoxicity) were also low in all patients. Five patients from Group 1 had a PR (partial response), 2 patients had CR (complete response), and 14 patients had stable disease. Seven patients from Group 2 showed progressive disease, 3 had a PR and 10 had stable disease. All immune parameters were significantly improved during treatment in Group 1 but remained unchanged or even deteriorated in Group 2. Administration of GM-CSF during treatment of cancer patients with conventional chemotherapeutic drugs results in a marked potentiation of deficient cellular immune responses *in vitro* and a change towards normalisation of cytokine serum levels. The results reported herein support the use of GM-CSF as immunopotentiator during chemotherapy, but more patients must be studied before definite conclusions can be drawn. © 1997 Published by Elsevier Science Ltd.

Key words: GM-CSF, chemotherapy, solid tumours, NK-LAK cytotoxicity, AMLR, cytokines, clinical response

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INTRODUCTION

GRANULOCYTE-MACROPHAGE COLONY-STIMULATING factor (GM-CSF) was originally described as a growth factor involved in myeloid cell proliferation and differentiation. Clinically, GM-CSF has been successfully used to accelerate leucocyte recovery in patients undergoing autologous bone marrow transplantation [1, 2]. Satisfactory haematological recovery has been reported by several investigators after administration of GM-CSF in patients with neutropenia due to aplastic anaemia [3] and in patients receiving chemotherapy [4].

GM-CSF is involved in host defence mechanisms and is a potent factor in activating macrophages for major histocompatibility complex (MHC) MHC-independent tumour cell killing [5]. Furthermore, GM-CSF has been shown to activate macrophages to increase MHC-class II (Iα) antigen expression [6], to secrete cytokines [7] and to inhibit the growth of pathogenic fungi [8]. Vaccination with irradiated tumour cells engineered to secrete GM-CSF has been shown to induce specific anti-tumour immunity [9]. Tumour infiltrating lymphocytes (TIL) from renal cell carcinoma are able to secrete GM-CSF upon stimulation with autologous tumour cells [10], and this ability has recently been shown to correlate positively with the clinical results after TIL immunotherapy in melanoma patients [11]. Herbelin and associates [12] reported that endogenous GM-CSF acts synergistically with IL-1 and IL-7 to stimulate thymocyte proliferation via an IL-2-independent pathway. In recent reports, GM-CSF was demonstrated to act synergistically with IL-2 on peripheral blood lymphocytes to induce cytotoxic cell populations [13–15] and to enhance autologous tumour cell killing among TIL from renal carcinoma [16]. In the present study, we investigated the efficacy of GM-CSF to improve immunological parameters in patients with solid tumours receiving second-line chemotherapy. The immunomodulatory effect of GM-CSF was then tested for correlation with clinical response to therapy.

PATIENTS AND METHODS

Patients

The study included 41 patients (21 men, 20 women, median age 61 years, range 42–73 years) with histologically proven malignant tumours. All had measurable metastatic disease which was refractory to standard first-line chemotherapy. They fulfilled the following criteria: Karnofsky performance status at least 80%; bilirubin level less than 1.7 mg/dl, creatinine level less than 2.2 mg/dl, leucocyte count higher than 3000/μl and platelet count higher than 100000/μl. They had not received any antineoplastic therapy during the 3 weeks preceding the onset of the study.

Patients were assigned to two groups. Group 1 included 21 patients (head and neck: 5; urogenital: 9; penis: 3; and colorectal cancers: 4) who were administered standard chemotherapy plus a four-cycle regimen of subcutaneous GM-CSF, 300 μg/day (Schering Plough, Alimos, Hellas). Group 2 included 20 patients (head and neck: 5, urogenital: 8; penis: 3; and colorectal cancers: 4) who were similarly treated, but with placebo instead of GM-CSF. The treatment protocol is shown in detail in Figure 1. The two groups were comparable with regard to age, performance status and dose intensity of previous therapy. 12 healthy volunteers (7 men, 5 women, median age 57 years, range 35–67 years) were also included in our studies. The patients were required to provide written informed consent and the study was approved by the review boards of participating institutions. A 50% or more shrinkage in the sum of the products of two perpendicular diameters of measurable lesions for at least 1 month was defined as a partial response (PR), with the complete disappearance of all laboratory parameters and clinically evaluable disease of at least 1 month duration constituting a complete response (CR) to therapy. A 0–50% decrease in the sum of measured diameters was defined as stable disease (SD) on physical examination and radiologic studies.

AMLR (autologous mixed lymphocyte reaction)

PBMC were isolated from heparinised peripheral blood by Ficoll-Hypaque density gradient centrifugation [17]. The AMLR was performed as previously described [18]. Briefly, 1×10^5 responder T lymphocytes isolated from PBMC were cultured for 6 days with 0.5×10^5 irradiated (33 Gy) autologous monocytes in 96-well round-bottomed plates (Costar, Cambridge, Massachusetts, U.S.A.) in 200 μl total volume of complete culture medium per well in a moist atmosphere of 5% CO_2, at 37°C. T lymphocytes were isolated as described previously [19]. Monocytes were isolated after incubation of PBMC on plastic Petri dishes [18]. Complete culture medium consisted of RPMI-1640 with Glutamex-I (Gibco, Grand Island, New York, U.S.A.) supplemented with 10% fetal calf serum (FCS) (Gibco) and 1% gentamycin. [^{3}H]-thymidine (1 μCi/well) (The Radiochemical Centre, Amersham, U.K.) was added 24 h prior to harvesting. All cultures were set up in triplicate.

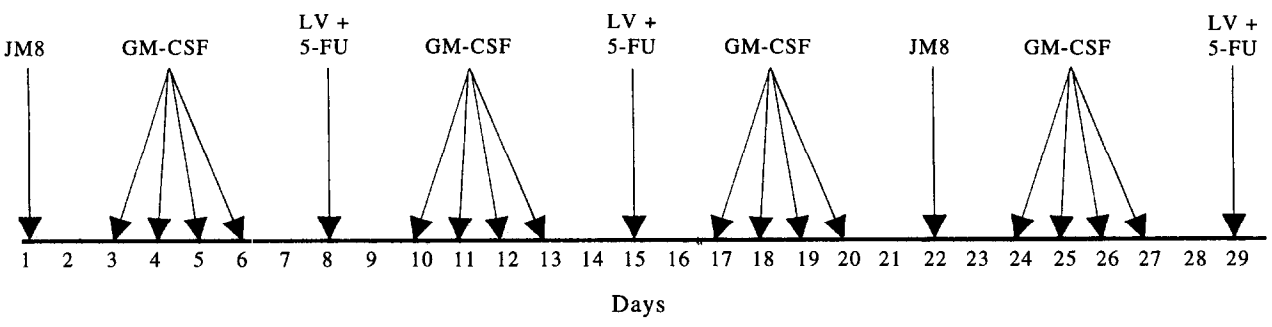

Figure 1. Treatment schedule. Carboplatin (JM8) 300 mg/m^2/day s.c. on days 1 and 22; leucovorin (LV) 200 mg/m^2/day s.c. and 5-fluorouracil (5-FU) 500 mg/m^2/day s.c. on days 8, 15 and 29; GM-CSF 300 μg/m^2/day s.c. on days 3–6, 10–13, 17–20 and 24–27. No drugs were given on days 2, 7, 9, 14, 16, 21, 23 and 28.

Cytotoxicity assays

NK (natural killer)-activity was tested in freshly isolated PBMC against the NK-sensitive K562 tumour targets. LAK-activity against NK-resistant Daudi tumour targets was tested in PBMC cultured with IL-2 as recently described [20]. Cytotoxicity assays for assessing NK- or LAK-activity were performed essentially as recently reported [21, 22]. Briefly, effector PBMC, freshly isolated or cultured with IL-2, were added in 100 μl aliquots (1×10^5 cells per aliquot) to microtitre plates (Costar). Tumour targets (10^7 cells) were labelled with 200 μCi of [^{51}Cr] sodium chromate (Amersham), washed to remove excess isotope, and added in 100 μl aliquots at 1×10^3 cells to the effector cells. After 4 h incubation at 37°C, 5% CO_2 and 95% humidity, 100 μl of supernatant were removed from each well for isotope counting in a gamma-counter (Packard, Downers Grove, Illinois, U.S.A.). Spontaneous release (targets in medium alone) and maximum release (targets incubated with 2% Triton-X) of isotope were also estimated. The percentage specific release of isotope was calculated as follows:

$$\% \text{ specific } {}^{51}\text{Cr release} = \frac{\text{c.p.m. test} - \text{c.p.m. spontaneous}}{\text{c.p.m. maximum} - \text{c.p.m. spontaneous}} \times 100$$

Quantitation of cytokine serum levels

Sera from patients were collected and stored at −70°C. Within the period of treatment (28 days), sera from healthy donors were also collected and stored at −70°C. All determinations were performed in duplicate and according to the manufacturer's instructions. ELISA kits specific for IL-1β and TNF-α were obtained from Endogen (Boston, Massachusetts, U.S.A.) for IL-2 from Genzyme (Boston, Massachusetts, U.S.A.), for IL-6 from Medgenix Diagnostics (Bruxelles, Belgium), and for PGE_2 from Advanced Magnetics Inc. (Cambridge, Massachusetts, U.S.A.).

AMLR, cytotoxicity and cytokine serum levels were assessed before treatment and one day after the end of each of the four-cycle injections with GM-CSF (Group 1) or placebo (Group 2).

Statistical analysis

Differences between mean values of groups were assessed by Student's *t*-test. Significance was presumed at $P < 0.05$.

RESULTS

Of the patients that were treated with standard chemotherapy plus GM-CSF (Group 1; $n = 21$), 2 had CR (10%), 5 had PR (24%) and 14/21 SD, giving an objective response rate (CR + PR) of 7/21 (33%). In Group 2, (same protocol without GM-CSF) there were 3 PRs (15%), 7 had progressive disease and 10 SD (15% objective responses).

PBMC from all patients with cancer exhibited markedly reduced proliferative responses *in vitro*, as compared to normal donors, when tested in the AMLR. As shown in Figure 2(a), before beginning treatment (day 0) patients from Groups 1 and 2 showed similar levels of AMLR responses (mean cpm: 5300 and 5700, respectively, compared to 21000 in normal controls; $P < 0.001$). During

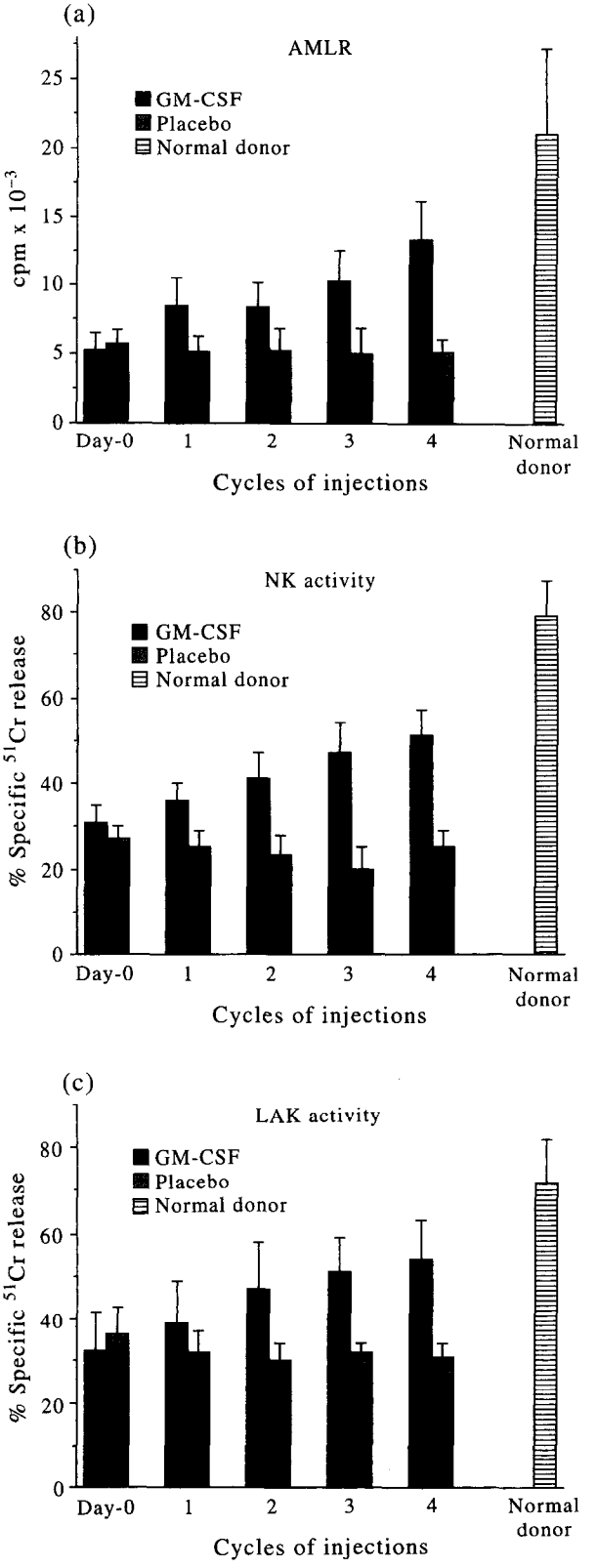

Figure 2. (a) AMLR responses, (b) NK-cell mediated cytotoxicity, (c) LAK-cell mediated cytotoxicity in patients receiving chemotherapy plus GM-CSF (Group 1; $n = 21$) in patients receiving chemotherapy plus placebo (Group 2; $n = 20$) and in normal donors ($n = 12$). All responses were evaluated before treatment (day 0) and during treatment (cycles 1–4) as described in Patients and Methods. Bars represent mean values ± S.D. from the pooled data. Normal donors were tested in parallel with the patients.

treatment, the AMLR responses in Group 1 gradually increased and reached 13300 cpm after the fourth cycle (day 28)(2.5-fold increase compared to day 0, $P < 0.001$; Figure 2(a)). In contrast, no significant changes were observed in Group 2 throughout the treatment. The NK activity (Figure 2(b)) of effector PBMC from these patients

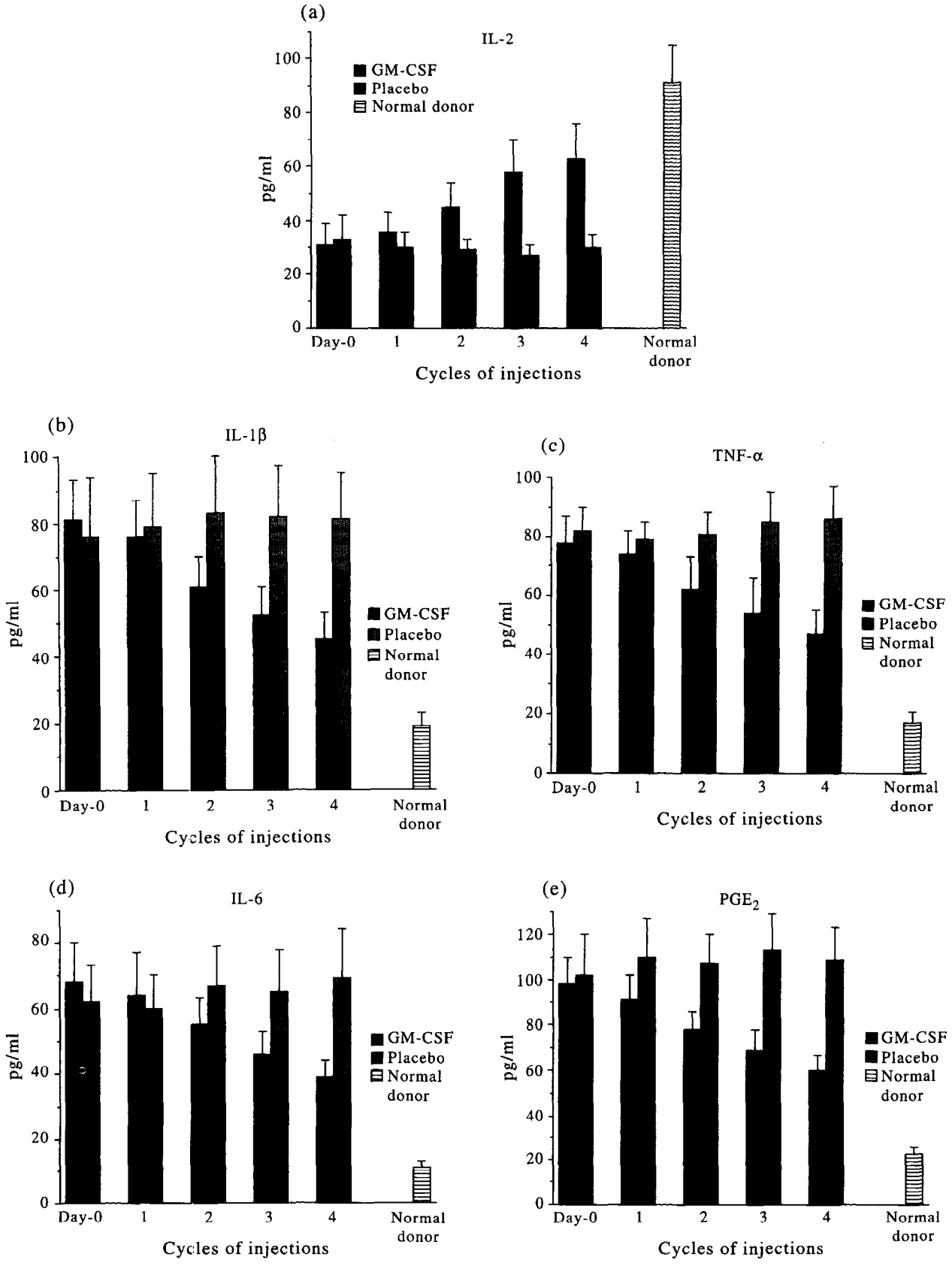

Figure 3. (a) Serum IL-2, (b) IL-1β, (c) TNF-α, (d) IL-6, (e) PGE_2 levels in patients with cancer (Groups 1 and 2) and in normal donors.

against K562 target cells decreased (32% and 27% on day 0 for Groups 1 and 2, respectively) compared to normal values (79%; $P < 0.001$). Patients from Group 1 showed an improvement in NK activity during treatment (51% cytotoxicity by the end of treatment; $P < 0.01$ compared to 32% on day 0), whereas no significant alterations were noticed among patients of Group 2. Similar cytotoxicity patterns were observed with IL-2 activated PBMC which exhibit LAK cytotoxicity (Figure 2(c)). As shown in Figure 2(c), mean LAK cytotoxicity in patients of Group 1 gradually increased during treatment: 32.5%, 39%, 47%, 51% and 54% by days 0, 7, 14, 21 and 28, respectively. LAK cytotoxicity among patients of Group 2 was even lower by the end of treatment, although this reduction was not statistically significant compared to day 0.

Cytokine levels *in vitro* have been shown to correlate with the extent of AMLR and cytotoxic responses [21–24]. In addition, cytokine serum levels have recently been described [25] to correlate with cellular immune responses *in vitro*. Thus, it was of interest to see whether the improvement in cellular responses achieved during treatment with GM-CSF in patients of Group 1 was followed by changes in cytokine serum levels. Mean IL-2 serum levels in patients of Groups 1 and 2 were greatly decreased (31 and 33 pg/ml, respectively) compared to healthy donors (91 pg/ml; $P < 0.01$) (Figure 3(a)). During treatment these levels were drastically increased in patients of Group 1 (mean by day 28: 63 pg/ml; $P < 0.01$) compared to the mean for day 0. In contrast to IL-2, on day 0 increased levels of IL-1β, TNF-α, IL-6, and PGE_2 were observed in cancer patients. Mean serum levels (pg/ml) in Groups 1 and 2 were, respectively, 81 and 76 for IL-1β, 78 and 82 for TNF-α, 68 and 62 for IL-6, and 98 and 102 for PGE_2. These values were significantly higher ($P < 0.001$) compared with those in normal donors (19 for IL-β, 17 for TNF-α, 11 for IL-6 and 23 for PGE_2 (pg/ml)). During treatment there was a gradual decrease in the levels of these cytokines in patients from Group 1 (Figure 3(b)–(e)). Thus, after the fourth-cycle of injections, mean IL-1β were decreased by 42% (47 pg/ml), TNF-α levels by 38% (48 pg/ml), IL-6 levels by 44% (38 pg/ml) and PGE_2 levels by 41% (58 pg/ml). In all cases, the levels of cytokines measured after the end of treatment (day 28) differed significantly ($P < 0.001$) compared to those before treatment (day 0). No significant changes could be noticed among patients from Group 2 (Figure 3(b)–(e)).

DISCUSSION

Our data demonstrate immunorestorative properties of GM-CSF in cancer patients undergoing chemotherapy which parallels clinical responses. Thus, 7 out of 21 patients from Group 1 (33%) who received GM-CSF during chemotherapy had objective responses (2 CR + 5 PR) and the rest (67%) showed SD, whereas 3 patients from the placebo group (Group 2) had a PR, 10 showed stable disease, and 7 had progressive disease.

Before treatment, all patients exhibited abnormal cytokine serum levels (decreased levels of IL-2 and increased levels of inflammatory cytokines IL-1β, TNF-α, IL-6 and PGE_2), which were associated with low lymphocyte responses *in vitro* measured either as proliferation in the AMLR (T cells) or as cytotoxicity (NK cells). Reduced IL-2 production *in vitro* has been reported in patients with various types of cancer with different histological origin [23, 26]. We have recently demonstrated that low IL-2 levels in culture supernatants of cancer patient-derived mononuclear cells are mainly due to the high levels of PGE_2, produced by monocytes, which inhibit IL-2 production *in vitro* [21, 27]. The negative effect of PGE_2 on IL-2 production has also been shown indirectly *in vivo*, where decreased serum levels of IL-2 in cancer patients who had undergone major surgery were associated with increased serum levels of PGE_2 [25]. In the same study [25], the serum levels of inflammatory cytokines IL-1β, TNF-α and IL-6 were also increased postoperatively. Of these cytokines, only IL-6, when measured at high levels in the sera of cancer patients, was shown to correlate with resistance to IL-2 therapy and poor survival [28]. Thus, although so far there is no direct evidence for an established role of these cytokines in negatively influencing IL-2 production *in vivo*, it seems, that at least in cancer patients, overproduction of the aforementioned cytokines *in vivo* is associated with decreased levels of IL-2.

Previous investigations demonstrated continually decreased AMLR and NK cell activity in patients with cancer [21, 23, 24, 29, 30] and consequently these assays gained increasing use in the clinical assessment of immunity. Such deficient responses were mainly attributed to inadequate levels of endogenously produced IL-2 [21, 23, 24, 27], low expression of IL-2-specific receptors on the responder (T cells) or effector (NK cells) lymphocytes and high endogenous PGE_2 production [21, 27]. A strict association between cytokine serum levels and the magnitude of AMLR levels, or NK cell-mediated cytotoxicity, was first demonstrated in cancer patients undergoing surgery, where intense changes in serum levels after surgery were followed by a more profound impairment in the AMLR levels, or NK cell function *in vitro* [25]. This was also confirmed in the present study where improvement of cytokine serum levels upon treatment with GM-CSF resulted in increased AMLR and NK cell responses in the same patients. Thus, it is evident that abnormal cytokine production *in vivo* influences negatively cellular immune responses *in vitro*. More importantly, these changes in cytokine serum levels may cause or exacerbate pre-existing immune dysfunctions which will apparently diminish the possibility of achieving satisfactory clinical results upon treatment with standard chemotherapeutic protocols.

However, it should be mentioned that the immune measurements in all GM-CSF treated patients were almost identical irrespective of the clinical results (CR + PR versus SD). This suggests that GM-CSF-induced restoration of immunological parameters does not always correlate with clinical improvement. Several reasons could account for this: first, immunological normalisation may in some cases, result in tumour bulk reduction followed by CR or PR, but in others this may not occur due to (i) lack of certain HLA haplotypes which would direct the killing of tumour cells by cytotoxic lymphocytes [31]; (ii) defects in the killing machinery of T and/or NK lymphocytes (e.g. reduced transcription rates of hydrolytic enzymes [32]) which may occur in individuals and may not be overcome by GM-CSF; and (iii) development of antigen-loss tumour variants which escape immune surveillance [33]. Second, immunological improvement may be secondary to tumour bulk reduction caused by GM-CSF plus standard chemotherapy. In this case, however, clinical responses would be seen only if GM-

CSF could additionally activate cells of the immune system to eliminate the autologous tumour cells. The inability of GM-CSF to induce tumoricidal activity *in vivo* (for the reasons outlined above) would result in no clinical responses despite immunological normalisation induced by the reduction of tumour load.

The mode of action of GM-CSF on lymphocytes *in vivo* remains unknown. The expression of GM-CSF-specific receptors on lymphocytes may be one possibility. Although the presence of a specific receptor for GM-CSF has been demonstrated in cell lines [34], the presence of the GM-CSF receptor on human lymphocytes has not yet been demonstrated [16, 35]. Since GM-CSF has been shown to activate macrophage/monocytes, to enhance non-specific and specific immune responses [5, 7], it might be speculated that GM-CSF acts indirectly on lymphocytes by stimulating the release of cytokines with lymphocyte-activating properties. Whether the increase in IL-2 serum levels represents a direct effect of GM-CSF or is an epiphenomenom caused by the GM-CSF-mediated decrease in PGE_2 serum levels remains to be explored. The decreased levels of IL-1β, TNF-α, IL-6 and PGE_2 could be the result of GM-CSF-mediated increased production of IL-10 or IFN-γ, which downregulate the production of these cytokines [36, 37]. All these possibilities are under investigation in our laboratory.

In summary, the treatrnent of cancer patients with standard chemotherapy and GM-CSF resulted in a 33.3% objective response with a parallel improvement in cytokine serum levels and cellular immune responses *in vitro*. Confirmation and extension of these studies may help to rationalise chemotherapeutic protocols combined with GM-CSF or other immunornodulators with improved therapeutic efficacy.

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