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Original Paper

Medroxyprogesterone Acetate Reduces the *In Vitro* Production of Cytokines and Serotonin Involved in Anorexia/cachexia and Emesis by Peripheral Blood Mononuclear Cells of Cancer Patients

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Medroxyprogesterone acetate (MPA) is widely used in oncology both in the treatment of hormone-related cancers and as supportive therapy in anorexia/cachexia syndrome (ACS), but conclusive data are not yet available to explain its anticachectic effect. ACS is characterised by weight loss, changes in metabolism, reduction of appetite, nausea and vomiting. Several cytokines, mainly interleukin (IL)-1, IL-2, IL-6 and tumour necrosis factor α (TNFα), are involved in the pathogenesis of ACS. Additionally, nausea and vomiting can be mediated by factors inducing serotonin (5-HT) production and/or release by pleiotropic cells including activated T lymphocytes. In the present study, we report the effect of MPA on peripheral blood mononuclear cells (PBMC) from 10 cancer patients in advanced stage of disease (6 head and neck, 2 colon, 1 lung and 1 ovary). The proliferative response of PBMC to PHA, anti-CD3 monoclonal antibody (MAb) or recombinant IL-2 (rIL-2), the production of IL-1β, IL-2, IL-6, TNFα and 5-HT by PHA-stimulated PBMC and the expression of lymphocyte membrane-bound IL-2 receptor (IL-2R) subunities (CD25 and CD122) were studied. The addition of MPA significantly reduced the PBMC proliferative response to PHA and anti-CD3 MAb but not to rIL-2. MPA 0.2 μg/ml was also capable of reducing the levels of IL-1β, IL-6, TNFα and 5-HT produced in culture by PHA-stimulated PBMC, whereas it did not induce any change in the percentage of PBMC expressing either CD25 or CD122 or both molecules after stimulation with PHA or anti-CD3 mAb.

Key words: anorexia/cachexia, cancer, cytokines, medroxyprogesterone acetate, serotonin

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INTRODUCTION

MEDROXYPROGESTERONE ACETATE (MPA) is a synthetic derivative of the natural steroid progesterone [1]. It is widely used in oncology both in the endocrine treatment of hormone-related cancers, such as breast and endometrial cancer [2], and as supportive therapy in anorexia/cachexia syndrome (ACS) [3], frequently found in advanced stage of disease. The ACS is a complex clinical condition characterised by asthenia, reduction of appetite, nausea and vomiting, weight loss, reduction of body fat and muscle tissue, changes of glucose and lipid metabolism, and enhancement of the side-effects of chemotherapeutic treatments [4, 5]. It has been reported that the administration of high doses of MPA, in the range of 1000–2000 mg daily, can improve the general condition of patients with ACS by increasing appetite, caloric intake and body weight, and by reducing nausea and vomiting and improving the performance status [6–8]. Presently, the main mechanisms involved in the antineoplastic activity of MPA are known, but conclusive data are not yet available to explain its anticachectic effect. Several studies support the hypothesis that some cytokines, derived

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from the immune system, mainly interleukin-1 (IL-1), IL-6 and tumour necrosis factor α (TNFα), play a crucial role in the pathogenesis of ACS [9]. Indeed, high serum levels of these cytokines have been previously reported in cancer patients in advanced stages and often cachectic [10, 11]. The administration of these cytokines induces *in vivo* the ACS [12], whereas treatment with the corresponding antibodies can reverse their effects [13, 14]. Further data, suggesting an involvement of the immune system in the onset of ACS, are provided by recent studies showing that activated lymphomonocytes are able to release serotonin (5-HT) [15], which is a mediator of emesis induced by different stimuli [16], and that 5-HT_{1A} receptors are present on the cell surface of activated T-lymphocytes [17].

Thus, since MPA has been reported to have an immunosuppressive effect on several immune functions *in vitro* [18], it may be hypothesised that MPA may counteract the factors inducing ACS, being able to reduce the production and/or release of the relevant cytokines and 5-HT from activated lymphomonocytes.

The aim of the present study was to evaluate whether MPA, at pharmacological doses *in vitro*, is able to influence the production and/or release of IL-1β, IL-2, IL-6, TNFα and 5-HT by peripheral blood mononuclear cells (PBMC) of advanced stage cancer patients stimulated by PHA. Furthermore, in order to confirm its immunosuppressive action, the effect of MPA on the *in vitro* proliferative response of PBMC to PHA, anti-CD3 monoclonal antibody (MAb) and recombinant IL-2 (rIL-2), was studied, as was the effect of MPA on the expression of membrane-bound IL-2 receptor (IL-2R) subunities, α (CD25) or β (CD122) chain or both, by PHA- or anti-CD3 MAb-stimulated PBMC of the same cancer patients.

MATERIALS AND METHODS

Peripheral blood mononuclear cells separation

PBMC from freshly drawn heparinised peripheral blood of 10 cancer patients in an advanced stage of disease (mean age 62.5 years, range 54–78, 8 males and 2 females) were studied: the patients' clinical characteristics are shown in Table 1. PBMC were separated by Lymphoprep, density gradient 1077 (Lymphoprep, Nycomed Pharma As, Oslo, Norway) and washed three times in Hank's solution (GIBCO, Paisley, U.K.). The cells were then counted and their viability tested using the Trypan blue dye-exclusion test. More than 95% of PBMC were viable. PBMC were kept in RPMI 1640 (GIBCO), supplemented with 20% autologous serum, 20 nM L-glutamine (GIBCO) and 10 μg/ml gentamicin (GIBCO), henceforth referred to as complete medium.

Mitogens and drug

To stimulate PBMC proliferation, we used PHA-M, anti-CD3 MAb and rIL-2 (all from Boehringer-Mannheim, Mannheim, Germany). PHA was diluted in RPMI 1640 medium and used at a concentration of 5 μg/ml. Anti-CD3 MAb was diluted in PBS (GIBCO) and used at a concentration of 5 μg/ml. Recombinant IL-2 was diluted in RPMI 1640 medium and used at a concentration of 200 IU/ml.

MPA (Pharmacia, Milan, Italy) was reconstituted in DMSO (Sigma, St Louis, Missouri, U.S.A.) at a concentration of 100 mg/ml and then diluted in RPMI 1640 medium to concentrations of 0.1, 0.2 and 0.4 μg/ml, respectively.

Proliferative response of PBMC to PHA, anti-CD3 MAb or rIL-2 ± MPA at different concentrations

PBMC proliferative response was evaluated using flat-bottomed 96-microwell microtitre plates (Costar, Cambridge, Massachusetts, U.S.A.). Each well contained 100 μl of a cell suspension of 1×10^6 cells/ml in complete medium, that is 1×10^5 cells/well, plus 100 μl of PHA (0.5 μg), anti-CD3 MAb (0.5 μg) or rIL-2 (20 IU). Fifty microlitres of MPA were added to the wells at the aforementioned concentrations. Control wells contained 1×10^5 cells in 125 μl of complete medium plus 125 μl of RPMI 1640 containing the different mitogens but MPA was not added. The cultures were set in triplicate and incubated at 37°C in 5% CO_2 atmosphere for 72 h. Fourteen to sixteen hours before harvesting, PBMC were pulsed with ^{3}H-thymidine (^{3}H-TdR) (Amersham International, Amersham, U.K.) at the concentration of 1 μCi/well in a volume of 20 μl/well. The cultures were harvested on glass fibre filters and washed with deionised water using a semi-automatic microwell harvester. The filters were counted for their radioactivity using a liquid scintillation β-counter and the results expressed as cpm.

Assay for IL-1β, IL-2, IL-6, TNFα and 5-HT production in cultures of PBMC stimulated with PHA ± MPA 0.2 μg/ml

The levels of IL-1β, IL-2, IL-6, TNFα and 5-HT were assessed in culture supernatants from PBMC stimulated with PHA in the presence or absence of MPA 0.2 μg/ml. This concentration was selected because it was the most effective in reducing the PBMC proliferative response to mitogens, which was approximately in the broad range of concentrations in plasma after the clinical administration of high-dose MPA (approximately 1000 mg/daily intramuscular or 1500–2000 mg/daily orally) [19–21].

Five hundred microlitres of PBMC suspension (1×10^6 cells/ml in complete medium) were stimulated for 24 h at 37°C in 5% CO_2 atmosphere. Centrifugation of the cultures

Table 1. Patients' clinical characteristics

Tumour site	Number of patients	Histological type	Stage	Performance status ECOG 0–1	Performance status ECOG 3–4
Head and neck	6	Squamous cell	IV	4	2
Oral cavity	3				
Oropharynx	2				
Hypopharynx	1				
Colon	2	Adenocarcinoma	IV	2	
Lung	1	Non-small cell	IV		1
Ovary	1	Serous Adenocarcinoma	IV	1	

was performed at 2000 rpm for 10 min to remove the cells and supernatants frozen at −20°C until assayed.

The detection of cytokines was performed with a sandwich ELISA test (Immunotech SA, Marseille, France), as previously reported [10]. Briefly, the samples were incubated in polystyrene microtitre strip wells coated with the first anticytokine MAb.

The cytokines captured by the first antibody were detected with a second MAb specific for each cytokine, linked to acetylcholinesterase and directed against a second epitope of the cytokine molecule. After removing the unbound enzyme-conjugated anticytokine MAb by washing, a chromogenic substrate solution was added to the wells. The extent of colour developed was proportional to the amount of cytokines present in the samples. The enzymatic reaction was stopped by adding an acidic solution. After 60–180 min, the absorbance at 405 nm for IL-1β, IL-2, IL-6 and at 450 nm for TNFα was measured with a spectrophotometer (Unidata, Rome, Italy). A standard curve was prepared by plotting the absorbance value of the standard cytokine versus corresponding concentrations (pg/ml). It should be noted that MPA does not interfere through cross-reaction with the cytokine assay nor through inhibition or promotion of binding of the antibody to specific cytokines. In fact, an additional standard curve was prepared with the addition of MPA 0.2 μg/ml: no differences were found between this standard curve and that without MPA (data not shown). The concentrations of cytokines in the samples tested were determined by extrapolation from the standard curve. The range of the assay was: 5–1000 pg/ml for IL-1β and IL-2; 2–1000 pg/ml for IL-6; 10–1200 pg/ml for TNFα. Intra-assay variations were 5% for IL-1β, 3% for IL-2 and IL-6, 6% for TNFα. Inter-assay variations were 7% for IL-1β and IL-2, 8% for IL-6 and 7% for TNFα. The results were expressed in pg/ml as mean ± standard error of mean (M ± SE).

Serotonin was evaluated with an ELISA test (Immunotech SA) based on the competition between 5-HT in the previously acylated sample and the 5-HT–enzyme conjugate, used as a tracer, for binding to the MAb coated on to the microtitre plate. Acylated samples and standards plus 5-HT–acetylcholinesterase were added to the wells, where they compete for a limited number of MAb-binding sites. After incubation, the content of the wells was aspirated and the wells washed to remove the excess of tracer. Enzymatic activity was measured by adding a chromogenic substrate and the absorbance of the resulting coloured solution was measured with a spectrophotometer. The concentration of 5-HT in each well was directly proportional to the corresponding absorbance value. The concentrations were determined by extrapolation from the standard curve. The range of the assay was 1.8–1000 nM; intra-assay variation was 9% and inter-assay variation was 8%. The results were expressed in nM as M ± SE.

Detection of membrane-bound IL-2 receptor, α (CD25) and β (CD122) subunities, on PBMC stimulated with PHA or anti-CD3 MAb ± MPA 0.2 μg/ml

After stimulation with PHA or anti-CD3 MAb, a variable percentage of T-lymphocytes undergoes blastic transformation that correlates with the surface expression of IL-2R, α and β subunities.

PBMC cultures (1×10^6 cells/ml in complete medium) were stimulated with PHA or anti-CD3 MAb and supplemented with MPA 0.2 μg/ml in the appropriate wells for 72 h at 37°C in a 5% CO_2 atmosphere. The cells were then washed twice in PBS solution and resuspended at a concentration of 2×10^6 cells/ml in RPMI 1640 medium. One hundred microlitres of this cell suspension was treated with 5 μl of phycoerythrin-conjugated anti-CD25 MAb (DAKO, Glostrup, Denmark) and 5 μl of fluorescein isothiocyanate-conjugated anti-CD122 MAb (Endogen, Cambridge, Massachusetts, U.S.A.). The cells were incubated at 4°C for 30 min, washed twice with PBS and resuspended in 500 μl of PBS. The expression of IL-2R, α (CD25) and β (CD122) subunities, was tested using dual colour flow cytometry (Ortho Cytoron, Ortho, Westwood, Massachusetts, U.S.A.). The results were evaluated as percentage of cells expressing CD25 or CD122 or both.

Statistical analysis

All results were expressed as M ± SE. Statistical analysis of the results was performed by using Student's two-tailed *t*-test.

RESULTS

Peripheral blood mononuclear cells' proliferative response ± MPA

The addition of MPA induced significant changes to the proliferative response to PHA and anti-CD3 MAb, but did

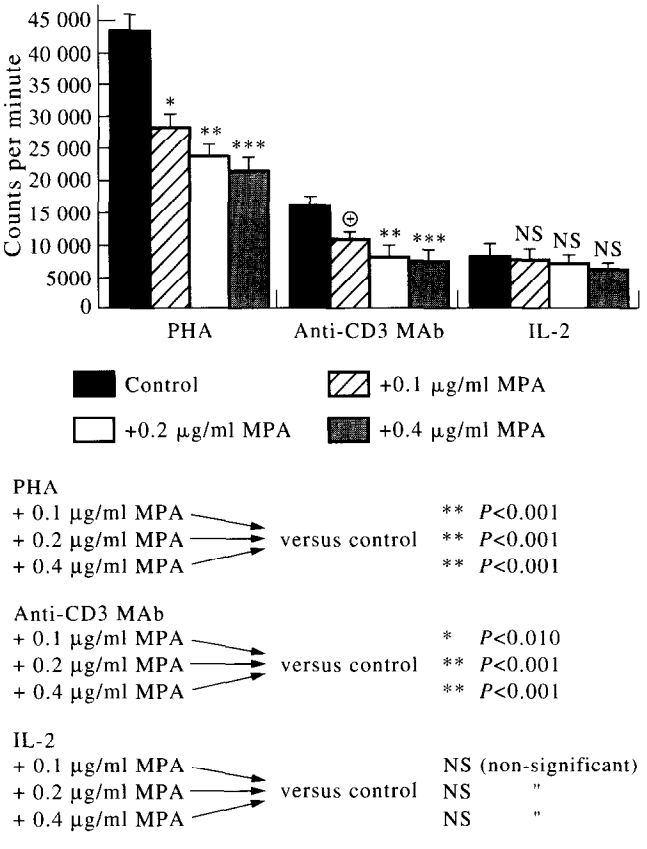

Figure 1. Proliferative response of PBMC from 10 cancer patients to PHA, anti-CD3 MAb or rIL-2 + MPA at different concentrations, as compared to PHA, anti-CD3 MAb or rIL-2 alone. The results are expressed in cpm, as mean ± standard error.

Table 2. (a) Cytokine and 5-HT production from PHA-stimulated PBMC of cancer patients and normal subjects; (b) cytokine and 5-HT production from cancer patients PBMC stimulated with PHA ± MPA 0.2 μg/ml. The results are expressed in pg/ml (nM for 5-HT), as mean ± standard error

Cytokines	Patient PBMC	*P*	Normal PBMC
a.			
IL1-β	3531 ± 317	<0.05	2470 ± 294
IL-2	2194 ± 228	<0.001	437 ± 54
IL-6	6528 ± 360	<0.001	2160 ± 36
TNFα	4469 ± 327	<0.001	945 ± 52
5-HT	1188 ± 183	0.001	75 ± 5
b.			
Cytokines	PHA	*P*	PHA + MPA 0.2 μg/ml
IL1-β	3531 ± 317	<0.05	2464 ± 337
IL-2	2194 ± 228	NS	1947 ± 107
IL-6	6528 ± 360	0.01	5123 ± 334
TNFα	4469 ± 327	<0.05	3142 ± 354
5-HT	1188 ± 183	<0.05	690 ± 107

NS, non-significant.

not induce changes in the response to rIL-2 (Figure 1). The reduction of the proliferative response to PHA and anti-CD3 MAb was dose-dependent so that a greater reduction was observed with the highest MPA dose used (0.4 μg/ml), whereas the proliferative response to rIL-2 was not affected by MPA addition (Figure 1).

Production of IL-β, IL-2, IL-6, TNFα and 5-HT in cultures of PBMC stimulated with PHA ± MPA

The production of IL-1β, IL-6, TNFα and 5-HT in culture supernatants from PHA-stimulated PBMC of 10 advanced stage cancer patients and 20 age-sex-matched normal healthy subjects was assayed. The levels were significantly higher in cancer patients than in controls (Table 2). The addition of MPA at 0.2 μg/ml significantly reduced the production of IL-1β, IL-6, TNFα and 5-HT but not IL-2 (Table 2).

Table 3. Expression of lymphocyte membrane-bound IL-2 receptor, α (CD25), β (CD122) and α/β subunities, on cancer patients PBMC stimulated with PHA or anti-CD3 MAb ± MPA 0.2 μg/ml. The results are expressed as a percentage of CD25+, CD122+ and CD25+/CD122+ cells, as mean ± standard error

	CD25+	CD122+	CD25+/CD122+
PHA	69 ± 5	61 ± 7	58 ± 4
P	NS	NS	NS
PHA + MPA 0.2 μg/ml	70 ± 8	58 ± 8	54 ± 6
Anti-CD3 MAb	71 ± 7	47 ± 8	32 ± 3
P	NS	NS	NS
Anti-CD3 MAb + MPA 0.2 μg/ml	65 ± 4	45 ± 6	29 ± 5

NS, non-significant.

Detection of membrane-bound IL-2 receptor, α (CD25) and β (CD122) subunities, on PBMC stimulated with PHA or anti-CD3 MAb ± MPA

The percentage of cells expressing membrane-bound IL-2 receptor, α (CD25) or β (CD122) subunities or both, on PBMC from 10 advanced stage cancer patients after stimulation with PHA or anti-CD3 MAb was not influenced by the addition of MPA at 0.2 μg/ml (Table 3).

DISCUSSION

The concentration of MPA we selected (0.2 μg/ml) as the most effective for our study may be reached *in vivo* following very high dose MPA administration (1500–2000 mg/day orally), such as that used for endocrine therapy of hormone-related cancers and, to a lesser extent, that used as supportive care for anorexia/cachexia in cancer patients, which is associated with the well-known related side-effects.

In a previous study, we and others [18] reported that MPA was able to reduce the proliferative response to PHA (but not to rIL-2) of PBMC from both normal female subjects and breast cancer patients, and that exogenous IL-2 was able to reverse this MPA-induced inhibition: this result suggested that a lower IL-2 production was the cause of reduced response.

The present study confirms our aforementioned results, and shows that the production in culture of IL-1β, IL-6, TNFα and 5-HT by PHA-stimulated PBMC is also significantly reduced in the presence of MPA. Direct evidence emerges both of the role played by some of these cytokines in the modulation of proliferation of immune competent cells and of the possible regulatory activity of MPA on the same immune competent cells.

Several studies have shown that MPA improves the general health status of patients with ACS by increasing weight, appetite and well-being [22], but the mechanisms underlying these effects are poorly understood. Previous reports suggest that certain cytokines produced and released by immune cells play an important role in the pathogenesis of ACS [9]. The levels of these cytokines are high in the serum of advanced stage cancer patients [10, 23] and are even higher in pleural and peritoneal neoplastic effusions, due to their production by tumour-associated lymphocytes (TAL) [11]. IL-1, IL-6 and TNFα are especially capable of inducing several metabolic effects. IL-1 can promote hyperglycaemia, by acting on pancreatic β cells [24], and anorexia due to its direct effect on the central nervous system which increases the release of corticotrophin-releasing factor [25]. IL-6 induces severe reduction of circulating fatty acids, hypoglycaemia, hypercalcaemia and weight loss [26–28]. Furthermore, it is known that TNFα inhibits the enzyme, lipoprotein lipase [29]: thus, by increasing circulating tryglicerides and reducing their incorporation in fat and muscle tissues, it enhances hepatic lipogenesis [30] and reduces protein content [31]. Almost all these metabolic changes are present in patients with ACS [32]. An association between serum levels of TNFα and the degree of ACS has been demonstrated in both animals [33, 34] and man [35]. Injection of TNFα induces weight loss, although it is, by itself, incapable of producing ACS, and anti-TNFα antibodies ameliorate ACS induced by this way [36].

We believe that the results of our present study may contribute to elucidating the pathogenetic role both of the cyto-

kines in the onset of ACS and of MPA in reversing many of their effects.

Other synthetic progestagens, such as megestrol acetate (MA) stimulate appetite and are able to inhibit the weight loss induced by TNFα [37]. MA inhibits intracellular levels of the messenger RNA for IL-1, which leads to the reduced synthesis and release of the correspondent cytokine [38]. In a recent study, we also reported that the high serum levels of IL-1α, IL-1β, IL-6, TNFα and soluble IL-2R, found in head and neck cancer patients in advanced stage, with a severe decrease (i.e. ≥10% of the ideal or customary) of body weight prior to treatment with MA, were reduced after MA treatment, with a statistically significant reduction of IL-1α, IL-1β and TNFα, whereas appetite and body weight were significantly increased [23].

A further interesting finding of our study is that MPA is able to inhibit 5-HT production and/or release by PBMC. Recently, it has been demonstrated that 5-HT, as well as cytokines, play an important role in upregulating T lymphocyte function *in vitro* and cell-mediated immunity *in vivo* [39]. It can, therefore, be hypothesised that, along with the cytokines, high amounts of 5-HT may be produced in advanced stage cancer patients as a consequence of the chronic activation of the immune system by specific (tumour antigens?) or non-specific stimuli. 5-HT is the main mediator of nausea and vomiting, which are common and distressing symptoms often associated with neoplasia itself and furthermore worsened by most antineoplastic treatments, such as cisplatin chemotherapy [40]: it is well known that 5-HT plays a key role in the onset of ACS [41]. The MPA-mediated inhibition of 5-HT production found in the present study may explain the previously reported effect of short-term administration of MPA on emesis induced by different combination chemotherapies [7].

The relationships between 5-HT and the cytokines involved in the pathogenesis of ACS still need to be clarified. Noteworthy, our results show that MPA is unable to interfere with both the activity of rIL-2 on lymphocytes and with IL-2R expression by the same cells.

In conclusion, our study provides further evidence that MPA, like MA, is able to hinder the activity of some cytokines, such as IL-1β, IL-6 and TNFα, which have a key role in the pathogenesis of ACS, by inhibiting their production and/or release. This mechanism may explain the clinical beneficial effect of MPA administration in cancer patients with ACS. A further interesting and, to our knowledge, not previously known finding highlighted by our study is the ability of MPA to inhibit 5-HT release by lymphocytes.

The broad spectrum of biological activities of 5-HT, including its role in chemotherapy-induced and non-chemotherapy-induced emesis, and its activity in the immune regulation of T lymphocyte function both *in vitro* and *in vivo*, suggests that further studies on the relationships between MPA and 5-HT production and/or release may help to clarify some important issues of not yet well understood clinical situations, such as emesis, ACS and immune impairment of advanced stage cancer patients.

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