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Cell Mediated Cytotoxicity and Cytokine Production in Peripheral Blood Mononuclear Cells of Glioma Patients

Clara M. Ausiello, Carla Palma, Alberto Maleci, Giulio C. Spagnoli, Carla Amici, Guido Antonelli, Carlo U. Casciani and Antonio Cassone

A mannoprotein preparation (MP) from *Candida albicans* induced MHC-unrestricted cytotoxicity in peripheral blood mononuclear cells (PBMC) from healthy subjects, but not in those from glioma-bearing subjects. The two groups of subjects did not significantly differ in the number of cells bearing typical natural killer (NK) markers (both in resting and MP stimulated PBMC) and NK activity. However, interferon gamma (IFN-γ) production was in tumour patients minimal or significantly reduced, as compared to healthy subjects, following PBMC stimulation by MP or phytohaemoagglutinin, respectively. In addition, minimal, if any, stimulation of interleukin-2 (IL-2) production was achieved in MP stimulated PBMC from glioma patients. Considering the pivotal role of the above cytokines in immune reponses, particularly in those concerning generation of antitumour effectors, our results consistently suggest that defective cytokine production is one possible mechanism of immunological impairment in glioma patients. They also provide indirect support for a possible clinical use of IFN-γ as an immunopotentiating agent in gliomatous subjects.
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INTRODUCTION

WE HAVE previously shown that *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) of healthy subjects with MP, a mannoprotein preparation derived from the human commensal microorganism *C. albicans*, induces lymphoproliferation, lymphokine production and activation of cytotoxic effector cells active against both NK sensitive and NK resistant targets, including uncultured neoplastic cells [1–3]. Potent immunomodulatory activities of candidal constituents have also been observed in normal and *Candida*-primed mice [4, 5].

To further explore the immunomodulatory potential of *C. albicans* in tumour-bearing subjects, we focused on the activity of MP in cultured PBMC from glioma patients, which has been reported to present distinct impairments of cell mediated immune responses [6–8]. In a preliminary report [9], we showed that MP-stimulated PBMC from glioma patients proliferate, but produce less interferon-gamma (IFN-γ) than PBMC from healthy controls. MP-induced generation of cytotoxic cells, but not generation of lymphokine-activated killer (LAK) cells [10, 11], was also greatly reduced in these patients [9]. We have now extended this investigation to examine number and function of natural killer (NK) precursors, as well as interleukin-2 and IFN-γ production by cultured PBMC of glioma patients, in response to MP or phytohaemoglutinin (PHA).

PATIENTS, MATERIALS AND METHODS

Patients

Table 1 reports clinical data concerning the 15 glioma patients enrolled in this study. All had a primary malignant glioma, and their Karnofsky performance status ranged from 80 to 100. All

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Table 1. Clinical data

Patient	Sex	Age	Tumour	
			Histology	Site
1	M	63	GM	Temporal right
2	F	74	GM	Parietal left
3	M	25	AA	Frontal right
4	M	58	GM	Parietal left
5	M	74	AA	Parietal left
6	F	64	AA	Temporal right
7	M	34	GM	Frontal right
8	F	61	AA	Frontal right
9	M	31	AA	Frontal left
10	M	41	AA	Frontal right
11	M	69	GM	Fronto-parietal right
12	F	57	GM	Temporo-parietal right
13	M	54	AA	Frontal right
14	M	60	AA	Parietal left
15	F	45	AA	Frontal right

AA = anaplastic astrocytoma, GM = glioblastoma multiformis.

were under supportive therapy with corticosteroids (betamethasone, 8 mg/day) and anticonvulsants (phenytoin, phenobarbital and mephobarbital, 50 mg/day). Mean duration of the pharmacological treatment at blood sample collection was 4 days (range 2–7 days) and patients had not undergone any antiblastic chemotherapy or radiotherapy. Age and sex-matched healthy subjects were used as controls.

Candida antigen and IL-2 preparations

The MP extract from the cell wall of *C. albicans* was obtained and chemically and antigenically characterised as described elsewhere [2, 12]. Recombinant IL-2 was kindly provided by Prof. G. Garotta (Hoffman–La Roche, Basel, Switzerland).

PBMC cultures

PBMC isolated by density gradient (Lymphoprep, Nyegaard, Oslo) from venous blood samples were washed in RPMI medium (Gibco, Grand Island, New York), resuspended in medium supplemented with 5% pooled AB serum and antibiotics (penicillin 100 IU/ml, streptomycin 0.1 mg/ml, Gibco, complete medium), and cultured in the presence or absence of MP (50 μg/ml), IL-2 (100 U/ml) or PHA (Wellcome, UK, 1.5 μg/ml) as described elsewhere [2, 3].

Cytotoxic assays

Fresh or cultured PBMC were washed twice in RPMI and used as effector cells in cytotoxic assay. In previous studies [3], it was determined that optimal cytotoxic response induced by MP or IL-2 occurred on the seventh day of culture. The NK sensitive K562 cell line was used as the target, and the conditions of radiolabel ($Na_2{}^{51}CrO_4$) release assay were those described elsewhere [3]. Percentages of specific lysis are reported as means of triplicate samples for each effector/target ratio with a standard error, which never exceeded 2%. One lytic unit (LU_{20}) was defined as the number of effectors required to produce 20% specific lysis of the target cells.

Cell staining and flow cytometry

The following monoclonal antibodies (MAb) directed against lymphocyte cluster determinants (CD) [13] were used: Leulla (CD16), Leu19 (CD56, NKH1), Leu5b(CD2) (Becton-Dickinson, Mountain View, California). Resting or activated PBMC were stained [3] with the indicated fluorochrome-labelled MAb. Flow cytometry was performed in a FACS Scanner (Becton Dickinson Immunocytometry Systems).

IL-2 and IFN-γ assays

Culture supernatants were harvested, on day 1 for PHA and on day 2 for MP stimulated cultures, and assayed for IL-2 content by using the IL-2 dependent murine T-cell line CTLL-2, as reported elsewhere [12]. Units/ml were calculated according to Gillis *et al.* [14] by using as internal test reference a rIL-2 preparation (Roche). Production of IL-2 which exceeded the 99% confidence interval of the mean amount of the cytokine detected in unstimulated PBMC cultures was considered as a positive response to stimulation with MP or PHA, both in normal and in glioma-bearing subjects.

Supernatants collected on day 3 for PHA and on day 7 for MP stimulated cultures were used to measure IFN-γ production. IFN titre was determined, and IFN characterised as IFN-γ by previously described assay [9]. IFN activity was expressed as international units (IU)/ml. Production of >10 IU/ml was considered as a positive response.

Statistical analysis

The significance of the differences of the means were analysed by the one-tailed Student's t test. The non-parametric Mann–Whitney U test was also used. Significance of the differences in responders/non-responders ratio to any treatment and between healthy donors and glioma patients was analysed by Fisher's exact test. Responders or non-responders were defined as outlined above.

RESULTS

NK activity and NK markers

MP induced non-MHC restricted cytotoxic activity in PBMC from normal subjects was seen to be generated from leucine-O-methyl ester sensitive cells bearing typical NK markers [3, 15]. Thus, it was of interest to examine whether the impaired generation of cytotoxicity following MP stimulation of PBMC from glioma subjects [9] could be related to low levels of NK activity or decreased number of cells bearing NK markers in gliomatous PBMC. As shown in Table 2, NK activity in PBMC from the 14 patients examined was not significantly lower than NK activity from an equal number of age and sex-matched healthy subjects. Confirming preliminary observations [9], the MP induced killer activity was greatly reduced in glioma patients as compared to healthy subjects [mean (S.E.) LU_{20} 19.2 (3.3) in healthy and 5.4 (1.6) in glioma subjects, respectively; $P = 0.001$, Mann–Whitney U test].

The presence of typical NK markers on PBMC from glioma patients was also sought for in comparison with PBMC from healthy subjects. Figure 1 shows that the mean percentage of cells bearing CD16, CD56 and CD2 markers did not significantly differ, either in resting or in MP activated PBMC, between patients and controls. No univocal modifications upon MP activation were recorded in the expression of CD16 and CD56 markers and no parallelism could be observed between the expression of classical killer cell markers and cytotoxic activities both in glioma patients and in healthy controls.

Cytokine production

Cytokines such as IL-2 and IFN-γ are involved in the generation of MHC unrestricted cytotoxic effectors [4, 16, 17]. As an

Table 2. NK activity of glioma patients and age and sex matched normal subjects (controls)

	Patients			Controls	
	% Killing	LU_{20}/ml		% Killing	LU_{20}/ml
1	16.2	2.4	1	12.2	0.9
2	8.4	0.6	2	50.4	29.1
3	38.7	15.0	3	38.2	11.4
4	30.5	19.4	4	60.4	20.5
5	28.7	7.0	5	42.3	13.3
6	12.4	0.5	6	32.5	7.7
7	31.8	10.8	7	44.6	14.0
8	49.2	21.8	8	50.1	7.4
9	55.6	23.3	9	87.8	56.3
10		ND	10	25.8	7.9
11	56.0	14.5	11	35.6	6.9
12	41.8	11.7	12	22.2	4.6
13	26.9	9.8	13	28.1	9.7
14	33.4	12.1	14	45.5	10.1
15	25.9	6.8			
Mean (S.E.)	32.5 (3.9)	11.1 (1.9)		41.1 (4.9)	14.2 (3.7)

NK activity was measured using freshly collected PBMC and K562 cells as target. Data are expressed both as % killing at an E:T ratio of 50:1 and lytic units/ml calculated on four different E:T ratios.
ND = not done.

impaired lymphokine cascade might account for the greatly diminished generation of cytotoxicty, the production of these lymphokines upon PBMC activation in glioma subjects was measured. As shown in Fig. 2 (panel A) the stimulation with MP induced a low but appreciable production (mean value 15.8 U/ml) of IL-2 in PBMC from normal subjects but not in those from glioma patients (mean value 5.4 U/ml, not significantly different from background IL-2 production in unstimulated PBMC from either category of subjects). No differences between the two groups of subjects were observed in the elevated production of IL-2 in PHA stimulated PBMC (Fig. 2, panel B). The lower IL-2 production in MP stimulated gliomatous PBMC was confirmed also in terms of responders/total subjects ratio (9/14 and 3/14 in normal and gliomatous subjects, respectively; $P < 0.05$, Fisher exact test). In the same subjects, an elevated production of IFN-γ was noticed following MP stimulation of

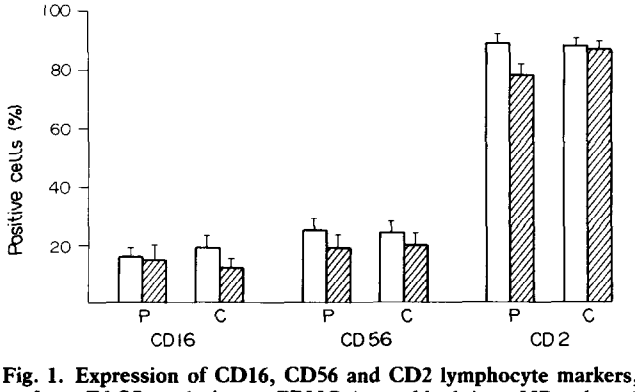

Fig. 1. Expression of CD16, CD56 and CD2 lymphocyte markers, as from FACS analysis, on PBMC (open blocks) or MP cultured PBMC (hatched blocks) in glioma-bearing (P) or normal (C) subjects. Data are given in % positive cells.

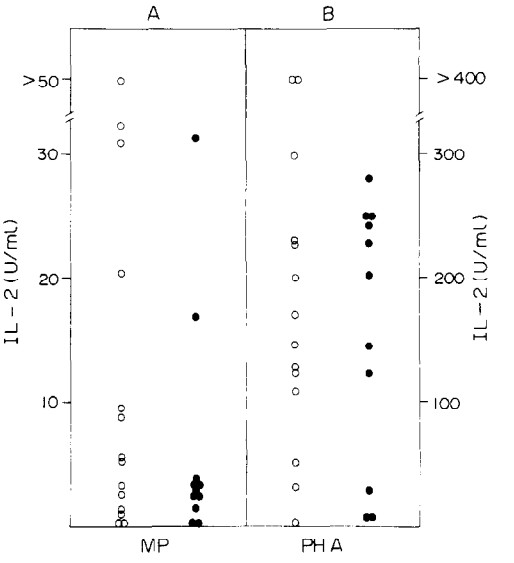

Fig. 2. IL-2 production in cultures of PBMC from healthy or gliomatous subjects stimulated with MP (panel A) or PHA (panel B).

PBMC from healthy controls [mean (S.E.) 410.7 (211)] and this production was highly depressed, if not abrogated, in glioma patients [9.3 (6.7)]. In fact, only 3 patients out of 15 could be qualified as responders, compared with 12 out of 14 controls ($P = 0.0021$, Fisher's exact test). Both patients' and controls' PBMC produced IFN-γ upon PHA stimulation, but the former produced a significantly lower amount of this lymphokine [mean (S.E.) 680 (208) and 195 (85) IU/ml in controls and patients, respectively].

DISCUSSION

Several cell mediated immune responses are depressed in glioma-bearing patients, in particular those concerning T-cell function, also in relation to the site of primary tumour and possible immunosuppressive effects of cytokines produced by gliomatous cells, in particular the transforming growth factor (TGF)β2 [6–8, 18].

Following a preliminary report [9] dealing with immune responses of PBMC from glioma-bearing patients to an immunomodulatory mannoprotein preparation (MP) of *C. albicans*, we have now investigated the mechanisms of this immune impairment in gliomatous subjects. Including (i) a diminished number or function of MP activatable precursor cells and (ii) a diminished production of those cytokines which are mostly involved in the generation activation of MHC unrestricted cytotoxic cells [10, 16, 17]. The data presented in this paper tend to rule out the former hypothesis as NK activity of PBMC was not significantly lower in our patients as compared to healthy subjects. Moreover, no differences between patients' and controls' PBMC were observed in the number of cells bearing classical NK surface markers such as CD16 and CD56, regardless of MP activation. Overall, no obvious correlation between ability to generate MP activated cytotoxicity and phenotype could be postulated.

Contrasting data have been reported on NK activity in glioma patients. Brown *et al.* [19] found a decreased NK activity in glioma patients with high grade malignancy. Ullen *et al.* [20], while finding a normal NK activity, observed that patients treated with corticosteroids present a decreased NK function.

All patients enrolled in our study had a highly malignant glioma, and were under supporting corticosteroid therapy before surgery. The average duration of this therapy, however, was rather short (4 days). To better analyse the effects of supporting therapy on NK activity 6 more patients affected by different pathology (2 meningioma, 1 sphenoidal sinus carcinoma and 3 intervertebral disk herniation) under steroid treatment were studied. These patients showed NK and MP induced cytotoxic activities in the normal range (data not shown).

Our data would rather support the concept of a defective cytokine production as a possible mechanism of impaired generation of antigen triggered, MHC unrestricted cytotoxicity in glioma patients. Production of IFN-γ has been shown to play a pivotal role in the induction or activation of LAK and T lymphocyte killer activity [16, 17, 21]. Furthermore, IFN-γ endogenously secreted during activation of tumour infiltrating lymphocytes with IL-2 and tumour necrosis factor was recently demonstrated [22]. Gemlo *et al.* [23] showed that the infusion of LAK plus rIL-2 is associated with increased serum levels of IFN-γ. The present data concerning 15 glioma-bearing patients add to those of a previous report dealing with 12 patients in showing clearly that the PBMC from these patients do not produce an appreciable quantity of IFN-γ when stimulated with candidal antigen, in a sharp contrast with the elevated IFN production seen in PBMC of healthy subjects. We also demonstrate here that PBMC from glioma patients produce less IFN-γ than normal PBMC upon stimulation with PHA. These findings suggest that impaired IFN-γ production might be a characteristic immunodeficiency of glioma patients, providing some indirect support to a possible clinical use of this cytokine as an immunopotentiating agent in these patients.

Another cytokine of great interest as anticancer immunomodulator is IL-2 [10, 11]. In the present study, MP stimulated a statistically significant increase of IL-2 production over that of unstimulated control in PBMC of normal subjects but not in those from glioma patients. Although there was a statistically significant difference even in the number of positive IL-2 responses between patients' and controls' groups, these data must be seen with caution because of the intrinsically low IL-2 amount detected in PBMC cultures of both gliomatous and control subjects in response to the antigen. In contrast with the data of Elliot *et al.* [24] we did not find a significantly diminished IL-2 production by PBMC of glioma patients following PHA stimulation.

Altogether, our findings suggest that the impaired cytotoxic response of PBMC from glioma patients upon stimulation with the microbial antigen could be functionally mediated by the diminished and/or abolished production of cytokines such as IFN-γ and IL-2. A possible relationship between these cytokines and immunosuppressive factor(s) released by glioma cells is being presently investigated. As $CD4^+$ helper/inducer T lymphocytes are a major source of both IL-2 and IFN-γ, possible alterations in the frequency of $CD4^+$ cells or $CD4^+/CD8^+$ ratios will also be examined.

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Prostate-specific Antigen: Problems in Analysis

Atilla Turkes, Jayne P. Nott and Keith Griffiths

We have compared an "in-house" Tenovus Institute prostate-specific antigen (PSA) assay with four different commercial kits (ELSA-PSA, IRMA-Count PSA, PROS-CHECK PSA and TANDEM-R PSA) that are available in the UK. There was only good correlation and linear regression parameters between the in-house assay and one of the kit methods. The difference in values for the same sample ranged from 2 to 100-fold. These discrepancies are due, in part, to the specificity of the polyclonal and monoclonal antibodies used in the procedures and the differing "hook effects" caused by the binding capacity of the antibody pairs in the immunometric assays. Discrepancies will, however, result from the differing potencies of the standards used for the calibration curves. This data highlights the urgency for the introduction of an internationally accepted reference standard for PSA.
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INTRODUCTION

PROSTATE-SPECIFIC ANTIGEN (PSA) is a recently isolated glycoprotein of prostatic origin [1] secreted by the epithelial cells into the seminal plasma. It is a serine protease, similar to kallikrein and thought to be responsible for the liquefaction of seminal coagulum [2, 3]. It is found on the endoplasmic reticulum and also in cytoplasmic vesicles and secretory granules of the normal, benign hypertropic and neoplastic prostate epithelial cells. The organ specific nature of this antigen stimulated research into its potential as a tumour marker and its determination has proved to be useful, not only for immunohistological identification of malignant tumours of prostatic origin [4, 5], but also for the determination of serum levels in the management of patients with prostatic cancer [6–8]. Comparative studies have also revealed that circulating PSA concentrations provide more information than prostatic acid phosphatase for the detection of current or future status of the disease [9–11].

The value of the determination of PSA in serum is becoming well established in the routine practice of general practitioners and urology clinics for the evaluation of patients with prostatic disease. The majority of these determinations are performed by commercially available immunoassay kits. As part of the Institute's prostate cancer research program, we have developed an "in-house" chemiluminometric assay (CLIA) for the determination of PSA in serum incorporating acridinium ester-labelled monoclonal antibody. We have compared the in-house CLIA with four different commercially available kits that are marketed in the UK.

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MATERIALS AND METHODS

Patients and samples

Patients with previously untreated metastatic carcinoma of the prostate participated in a phase III randomised trial [12, 13]. All patients had histologically proven carcinoma of the prostate with metastases and received first line endocrine treatment of either orchidectomy or a monthly injection of a depot luteinising hormone-releasing hormone analogue (goserelin). Serum samples were collected from patients at different stages of their treatment and stored at −20°C or lower until assayed.

Purification of PSA

PSA was purified from seminal plasma by a procedure similar to that described by Wang and his colleagues [14]. A pool of seminal plasma (40 ml) was ammonium sulphate fractionated, dialysed and applied to an ion-exchange column (1.6 × 40 cm) packed with DEAE Sepharose CL-6B (Pharmacia-LKB Biotechnology). The PSA was eluted from the column by a linear salt (NaCl) gradient and fractions containing PSA were pooled, concentrated and subjected to gel exclusion column (2.6 × 90 cm) chromatography on Sephacryl S-200 (Pharmacia–LKB Biotechnology). Following elution of the column with Tris-HCl (0.01 mol/l, pH 8) buffer, fractions containing PSA were pooled, concentrated and applied to a DEAE Sepharose CL-6B column. This time PSA was eluted by pH gradient and PSA fractions, following further gel exclusion chromatography on Sephacryl S-200, were pooled and the final protein concentration was determined (BCA protein assay reagent, Pierce). Each purification run yielded 3–5 mg PSA and the purity of the protein was >95% on polyacrylamide gel electrophoresis.

Antisera

Sheep, each 8–10 months old, were immunised by multiple injections of purified PSA (200 μg). The primary immunisation