Cancer Immunol Immunother (1989) 29: 237–241

Cancer Immunology Immunotherapy

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Augmentation of the generation of lymphokine-activated killer cells after a single dose of mitomycin C in cancer patients

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Summary. The effect of mitomycin C administration on the generation of cytotoxic cells, induced by in vitro activation of peripheral blood mononuclear cells (PBM) with interleukin-2, was studied in patients with various carcinomas. The ability of PBM to generate lymphokine-activated killer (LAK) cell activity against Raji cell targets was significantly augmented 5 and 7 days after a single intravenous dose of 12 mg/m^2 mitomycin C, when compared to that of PBM obtained before mitomycin C injection. Further, LAK cell activity against autologous tumor cells was also significantly increased after the drug administration. The distribution of lymphocyte subsets exhibited a significant increase in the percentage of CD3$^+$ cells after injection, with the elevation of the CD4/CD8 ratio. Furthermore, the proportion of the CD4$^+$ Leu8$^+$ subpopulation, which identifies inducers of suppression, was significantly reduced. Thus, the decrease in the proportion of suppressor-inducer subsets of PBM might be at least partially, responsible for the augmented generation of LAK cells after mitomycin C administration.

Introduction

Depending on conditions, several anticancer drugs have been reported to affect particular immune functions selectively, ultimately resulting in augmentation of the immune response. The generation of cytotoxic cells has been shown to be augmented in mice and humans after treatment with these drugs [2, 8, 17, 24], and the immunomodulating effects of mitomycin C have been reported by several authors [1, 16, 20].

Recently, activation of peripheral blood mononuclear cells (PBM) with interleukin-2 (IL-2) has been shown to induce cytotoxic cells, which lyse both natural-killer-resistant and -sensitive tumor cell lines in addition to freshly isolated allogeneic and autologous tumor cells [9, 11]. Further, it has been reported that the systemic administration of these lymphokine-activated killer (LAK) cells in conjunction with IL-2 is capable of mediating the regression of established metastatic tumor in patients with advanced cancer [18, 19]. It has been shown subsequently that high-dose administration of IL-2 alone also induces tumor regression in patients with certain types of advanced cancer [15, 18].

In the present study, we examined the effects of mitomycin C administration to cancer patients by following the development of LAK cells from PBM in culture. The results indicated an augmenting effect of mitomycin C, and the changes in the phenotypic pattern in PBM following injection were, therefore, further investigated.

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Materials and methods

Peripheral blood mononuclear cells. A group of 36 patients with various carcinomas, including 17 with gastric carcinoma, 15 with colorectal carcinoma, and 4 with breast carcinoma, were given a single intravenous dose of 12 mg/m^2 mitomycin C. Peripheral blood samples were obtained serially from 23 of these patients before and 3, 5, and 7 days after mitomycin C administration. Of these, LAK cells were generated in 11 patients and natural killer activity was measured in 10 patients. Both LAK cell and natural killer activity were studied in 2 patients. In 13 patients, the blood samples were taken before and 5 days after the drug injection. PBM were isolated by Ficoll/Hypaque density gradient sedimentation. The cells were suspended in RPMI 1640 medium containing 10% pooled human AB serum, supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium).

Preparation of autologous tumor cell targets. Single-cell suspensions of solid tumors were prepared by the method described by Grimm et al. [10] with some modifications. Briefly, surgical specimens of tumor tissue were minced with a scalpel and dissociated by mechanical stirring for 60 min in RPMI 1640 medium containing 0.01% hyaluronidase, 0.8% collagenase and 0.002% DNase at 37° C. The resulting cell suspension was centrifuged on a 100% Ficoll/Hypaque gradient for 30 min at 750 *g* to eliminate dead cells or erythrocytes. After washing, cell suspensions were then overlaid on a discontinuous three-step gradient of Ficoll/Hypaque (25%, 75% and 100%) followed by centrifugation at 250 *g* for 20 min. The tumor cells were collected from the 25%–75% interface, washed, checked for viability, and counted. Tumor cells were cryopreserved in 90% human serum and 10% dimethyl-sulfoxide by controlled-rate freezing. Immediately prior to the cytotoxicity assay, the cells were thawed and checked for viability.

Table 1. Changes in the generation of lymphokine-activated killer (LAK) cell activity against Raji cells and in the NK activity following a single dose of mitomycin C administration

Time after mitomycin C administration (days)	LAK activity against Raji cells ($n = 13$)				Natural killer activity[c] ($n = 12$)	
	PBM + IL-2[a]		PBM alone[b]			
	Mean ± SE	Range (median)	Mean ± SE	Range (median)	Mean ± SE	Range (median)
0[d]	29 ± 5	12–63 (26)	3 ± 2	0–5 (3)	28 ± 4	15–43 (30)
3	33 ± 4	13–65 (29)	4 ± 2	0–7 (3)	26 ± 4	19–33 (28)
5	50 ± 7[e]	14–81 (48)	4 ± 1	1–6 (4)	29 ± 3	19–41 (27)
7	39 ± 6[f]	16–73 (35)	3 ± 1	0–6 (2)	28 ± 2	24–38 (26)

[a] Peripheral blood mononuclear cells (PBM) were cultured in the medium containing 1 U/ml recombinant interleukin-2 (IL-2), and induced cytotoxicity against Raji cells was measured at an E : T ratio of 40 : 1

[b] PBM were cultured in the medium alone and the cytotoxicity against Raji cells was measured at an E : T ratio of 40 : 1

[c] NK activity was measured at an E : T ratio of 20 : 1

[d] Before mitomycin C administration

[e] $P < 0.01$, [f] $P < 0.05$, by the Wilcoxon ranks test for paired samples, when compared to the value before mitomycin C administration

Preparation of effector cells. PBM at a concentration of 1×10^6/ml were cultured for 4 days in complete medium containing 1 U/ml recombinant IL-2 for preparation of LAK cells. The recombinant IL-2 was prepared at Takeda Pharmaceutical Co. (Osaka, Japan) and had a specific activity of 3.5×10^4 U/ml as assayed on IL-2-dependent murine NKC3 cells [12]. The specific activity of recombinant IL-2 per unit was almost 300 times higher than that used in other studies. Control cultures, containing PBM alone, were routinely tested for background cytotoxicity. At the end of the incubation, the cells were harvested, washed three times and resuspended in the complete medium for cytotoxicity assay.

Cytotoxicity assay. The cytotoxicity of cells harvested from cultures or of PBM was determined in a standard 4-h ^{51}Cr-release assay. Target cells consisted of the myeloid cell line K-562 for natural killer activity, the B-lymphoblastoid cell line Raji and autologous tumor cells. The target cells were radiolabeled with 100 μCi sodium [^{51}Cr] chromate for 1 h at 37° C. Effector cells were added to each of four replicate round-bottom microculture wells. Then 1×10^4 ^{51}Cr-labeled target cells were added to each well containing the effector cells, to six wells containing medium alone (to determine spontaneous release) and to six wells containing detergent (to determine maximal release). After a 4-h incubation period, the release of ^{51}Cr label was measured with a Titertek supernatant collection system, and quantified in an automated gamma counter. The percentage of specific ^{51}Cr release was calculated as follows:

$$\text{Lysis (\%)} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

Flow cytometry. Analysis of surface markers of PBM by monoclonal antibodies was performed according to the method of Stephan et al. [21]. Briefly, peripheral blood was treated with fluorescein-conjugated monoclonal antibody and incubated on ice with gentle shaking for 15 min. The erythrocytes in this mixture were then removed by osmotic shock using an isotonic NH_4Cl solution. After 10 min, analysis of the fluorescence staining of cells was performed by an Ortho Spectrum III laser flow cytometry system. Monoclonal antibodies used for phenotyping included OKT3(CD3), OKT4(CD4), OKT8(CD8), OKT11(CD2), OKM1(CD11b), B-1(CD20), OKIal, Leu2a(CD8), Leu-3a(CD4), Leu7, Leu8, Leu11(CD16), and Leu15(CD11). Two-color staining analyses were also performed.

Analysis of the data. For each patient and for each parameter, we calculated the change between the pretreatment sample and each posttreatment sample. Parametric statistics (mean and standard errors of the mean), and median and range were used for descriptive purposes, and a nonparametric method (Wilcoxon ranks test for paired samples) was used to calculate the significance of difference between the parameters of the samples in the two groups.

Results

Effect of mitomycin C administration on the generation of LAK cells

The ability of PBM from cancer patients to generate LAK cell activity against Raji cell targets was assayed before and, 3, 5, and 7 days after mitomycin C injection. As shown in Table 1, the generation of LAK cells in PBM was significantly increased 5 and 7 days after the drug administration, when compared to that before treatment ($P < 0.01$ and $P < 0.05$, respectively). The peak level of the cytotoxic activity was observed 5 days after injection. There was no detectable generation of cytotoxic cells nor significant change in the level of cytotoxicity, when PBM were cultured with the medium alone. Natural killer activity in PBM following the drug treatment did not differ significantly from the value before mitomycin C administration. The capacity of PBM from cancer patients to induce LAK cell activity against autologous tumor cells was also measured before and 5 days after mitomycin C administration. The cytotoxic activity observed 5 days after the drug injection was significantly augmented as compared to that before treatment (Table 2). A significant increase in LAK cell activity against Raji cell targets was also observed when the cytotoxicity was assayed at the same time. In these patients, total leukocyte counts were not significantly decreased 5 days after mitomycin C injection (5750 ± 520),

Table 2. Changes in the generation of LAK cell activity against autologous tumor cells and Raji cells after mitomycin C (MMC) administration

Patient no.	Disease	Autologous tumor cells[a]		Raji cells[b]	
		Before MMC administration	5 days after MMC administration[c]	Before MMC administration	5 days after MMC administration[c]
1	Colon carcinoma	9	20	46	72
2	Colon carcinoma	15	28	50	69
3	Gastric carcinoma	10	11	27	35
4	Colon carcinoma	30	27	37	38
5	Colon carcinoma	2	34	18	46
6	Gastric carcinoma	38	43	39	56
7	Gastric carcinoma	12	17	23	36

[a] PBM were cultured in the medium containing 1 U/ml recombinant IL-2, and induced cytotoxicity against autologous tumor cells was measured at an E : T ratio of 40 : 1

[b] PBM were cultured in the medium containing 1 U/ml recombinant IL-2, and induced cytotoxicity against Raji cells was measured at an E : T ratio of 40 : 1

[c] $P < 0.05$, by Wilcoxon ranks test for paired samples, when compared to the value before mitomycin C administration

Table 3. Changes in lymphocyte subsets following mitomycin C (MMC) administration

Lymphocyte subsets	Percentage of lymphocyte subsets (%) ($n = 8$)			
	Before MMC administration		5 days after MMC administration	
	Mean ± SE	Range (median)	Mean ± SE	Range (median)
CD2	77.8±3.2	65–89 (79)	77.6±3.6	64–86 (82)
CD3	58.6±4.5	48–82 (57)	65.2±2.4[a]	60–77 (65)
CD4	33.7±2.1	20–42 (36)	36.4±3.6	20–46 (40)
CD8	33.3±2.1	29–39 (30)	31.1±2.0	21–36 (31)
CD11b	20.2±6.8	2–51 (15)	25.4±6.4	3–45 (25)
Leu7	31.2±3.1	23–46 (29)	21.1±2.9[b]	13–32 (21)
CD16	19.5±4.8	1–30 (23)	16.4±4.3	6–37 (14)
CD20	15.8±4.9	4–37 (15)	14.4±2.7	5–25 (12)
OKIa1	32.4±6.0	18–55 (32)	32.7±6.1	17–55 (27)

[a] $P < 0.05$, [b] $P < 0.01$, by Wilcoxon ranks test for paired samples, when compared to the value before mitomycin C administration

compared to those before treatment (5560±580), nor were the lymphocyte counts significantly reduced (910±78 and 890±118, respectively).

Phenotypic analysis of PBM after mitomycin C administration

To examine the changes in the phenotypic pattern of PBM after mitomycin C administration, PBM were obtained from the patients before and 5 days after the drug injection. The proportion of $CD3^+$ cells in PBM after treatment was significantly increased as compared to that before injection. The distribution of T cell subsets after injection showed a slight increase in the proportion of $CD4^+$ cells, whereas there was a slight decrease in the percentage of $CD8^+$ cells. Thus, the CD4/CD8 ratio was significantly increased. The percentage of $Leu7^+$ cells was significantly reduced, although the proportion of $CD16^+$ cells was not significantly altered. No significant difference was observed in the proportions of $CD2^+$, $CD11b^+$, $CD20^+$ and $OKIa1^+$ cells (Table 3). Further, subpopulations of $CD4^+$ and $CD8^+$ T cells in PBM obtained before and 5 days after mitomycin C administration were analysed using two-color flow cytometry. There were no significant changes in the percentages of $CD4^+$ $Leu8^-$, $CD8^+$ $CD11^+$, and $CD8^+$ $CD11^-$ cells following the drug injection. However, mitomycin C administration resulted in a significant fall in the proportion of $CD4^+$ T cells expressing the Leu8 antigen, which identifies inducers of suppression (Table 4).

Discussion

Certain anticancer drugs have been reported to enhance the generation of cell-mediated cytotoxicity in mice and humans under defined conditions. Treatment of mice with Adriamycin resulted in selective augmentation of a cell-mediated cytotoxic response [8, 17, 24]. Our previous study showed that the capacity of PBM to be converted to cell-mediated cytotoxic cells in cancer patients was significantly augmented after a single dose of Adriamycin [2]. Further, we examined the immunomodulating effects of mitomycin C on the cytotoxic response and found that its direct addition to primary stimulation cultures augmented the development of the cytotoxic response of PBM to allogeneic lymphoblastoid cells [1]. In the present study, we demonstrated that the ability of PBM to generate LAK cells was significantly increased after a single dose of mitomycin C injected into cancer patients.

Table 4. Changes in subpopulations of $CD4^+$ and $CD8^+$ T cells after mitomycin C (MMC) administration

Subsets	Percentage of subpopulations (%) ($n = 9$)			
	Before MMC administration		5 days after MMC administration	
	Mean ± SE	Range (median)	Mean ± SE	Range (median)
$CD4^+$ $Leu8^+$	11.5±1.8	4–19 (12)	8.2±2.1[a]	2–21 (7)
$CD4^+$ $Leu8^-$	25.9±3.0	18–46 (23)	25.4±3.2	15–38 (24)
$CD8^+$ $CD11^+$	4.7±0.9	1– 9 (5)	4.0±1.2	1–11 (4)
$CD8^+$ $CD11^-$	15.9±3.8	9–41 (14)	14.0±1.8	8–20 (15)

[a] $P < 0.05$, by Wilcoxon ranks test for paired samples, when compared to the value before mitomycin C administration

In an experimental system of rats and mice, it was reported that a single injection of mitomycin C into the peritoneal cavity of the animals resulted in enhancement of tumoricidal activity of the peritoneal exudate cells. This activity was subsequently found to be associated with the plastic-adherent, esterase-positive peritoneal macrophages [16, 20]. In our study, it was unlikely that the augmentation of the cytotoxicity was due to the direct activation of monocytes in PBM, since the natural killer activity of PBM was not increased; in addition, the cytotoxicity was not detectable when PBM, obtained after mitomycin C administration, were cultured in the medium alone.

The effects of anticancer drugs on human lymphocyte subsets have been reported by several investigators [3, 4]. Previously, we found that the distribution of T cell subsets in PBM from cancer patients after Adriamycin administration showed a significant increase of $CD8^+$ cells, while no significant difference was observed in the $CD4^+$ populations [2]. However, the present results demonstrated that, after mitomycin C injection, the percentage of $CD3^+$ cells in PBM was significantly increased with the elevation of the CD4/CD8 ratio. Further, the proportion of $Leu7^+$ cells was significantly reduced.

The phenotypes of LAK cells from PBM have been described as $CD3^+$, $CD8^+$, generated from $CD3^-$, $CD16^-$, $Leu7^-$ precursors [11]; it has also been suggested that $CD16^+$ cells are LAK precursors [13]. However, Damle et al. [7] demonstrated that, by using both positive and negative selection techniques, LAK activity could be generated in each of the following: $CD4^+$, $CD8^+$, $CD16^+$, $CD20^+$, and the $CD2^-$, $CD16^-$, $CD20^-$ subpopulation of human lymphocytes, with the exception of $Leu7^+$ T cells. Thus, LAK activity could be exhibited by cells that were both functionally and phenotypically heterogeneous. With regard to LAK precursors, the augmented generation of LAK cells observed in this study could not be simply explained in terms of the phenotypic pattern of lymphocytes in PBM after injection of mitomycin C.

Recently, $CD4^+$ and $CD8^+$ T cells have been subdivided on the basis of expression of surface antigens: Leu8 [6] and CD11 [14]. Berd and Mastrangelo [5] reported that treatment of melanoma patients with cyclophosphamide resulted in a progressive fall in the proportion of $CD4^+$ T cells expressing the CD45 antigen, which identifies inducers of suppression. However, there were no significant changes in the percentages of $CD4^+$, $CD8^+$ cells, or the subpopulation of $CD8^+$ cells expressing CD11, the marker of suppressor cells. The depletion of the suppressor-inducer subset could, therefore, be at least partially responsible for the immunomodulating effects of cyclophosphamide. It was shown that murine spleen cells cultured with increasing amounts of IL-2 induced suppressor cells that were characterized as classic suppressor T cells and suppressed the lymphokine-induced cytotoxicity [22, 23]. The present results demonstrated that mitomycin C administration induced a significant decrease in the proportion of $CD4^+$ $Leu8^+$ cells, without the depletion of $CD8^+$ $CD11^+$ suppressor T cells. It has been shown that a T cell with the phenotype of $CD4^+$ $Leu8^+$ is not a suppressor, but is necessary for the induction of $CD8^+$ suppressors [6]. Thus, the augmentation of the generation of LAK cells after mitomycin C administration might be at least partly related to the reduction in the proportion of $CD8^+$ $Leu8^+$ suppressor-inducer T cells.

LAK cells have been shown to exhibit a high degree of antitumor reactivity, as shown in vitro by the capacity to lyse a wide variety of fresh noncultured tumor cells [9, 11]. It has also been reported that combined therapy with LAK cells and IL-2 is capable of causing the regression of established metastatic tumor in patients with advanced cancer [18, 19]. Further, very high doses of IL-2 alone can mediate objective regressions in patients with melanoma or renal cancer [18, 19]. Our findings, in which the capacity of PBM from cancer patients to induce LAK cells, even against autologous tumor cells, was significantly augmented after a single injected dose of mitomycin C, may allow the combination of optimal mitomycin C administration with this type of immunotherapy in the treatment of cancer patients.

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Received 7 May 1988/Accepted 31 January 1989