Cancer Immunol Immunother (1987) 24: 259–262

Cancer Immunology Immunotherapy



# Augmentation of the generation of cell-mediated cytotoxicity in culture by mitomycin C

**Tsuyoshi Akiyoshi, Shinya Arinaga, and Hideo Tsuji**

Department of Surgery, Medical Institute of Bioregulation, Kyushu University 69, Beppu 874, Japan

**Summary.** The effects of mitomycin C (MMC) on the generation of cell-mediated cytotoxicity in primary stimulation culture of human peripheral blood mononuclear cells (PBM) with the B lymphoblastoid Raji cell line were assessed. The cell-mediated cytotoxicity induced in culture was significantly augmented when MMC was added to cultures on day −1 to day 3 for 24 h at concentrations of $2.5 \times 10^{-2}$ μg/ml and $2.5 \times 10^{-3}$ μg/ml. To identify the cell populations affected by MMC, PBM were separated by adherence to plastic after treatment with MMC for 24 h (day −1). The two populations were recombined with untreated separated cells and stimulated with antigen. The ability to develop an augmented cell-mediated cytotoxicity was associated with the adherent cell fraction of MMC-treated PBM. Therefore, the ability of MMC-treated adherent cells to produce interleukin 1 (IL 1) was examined. Significantly higher levels of IL 1 were produced by treated cells as compared to untreated adherent cells. The results appear to indicate that the selective effects of MMC on the adherent cell fraction, especially the modification of IL 1 production, may be involved in the mechanisms of MMC-induced augmented cell-mediated cytotoxicity.

## Introduction

It has been demostrated that certain anticancer drugs induce selective modulating effects on immune responses. Under defined conditions, the immunopotentiating effects of these drugs on cytotoxic T cell activity have been reported [4, 7, 10, 11]. Also, several studies have shown that the drug-induced changes in the production or release of soluble mediators of the immune response, interleukins, may play an important role in modulating the cellular responses [2, 3, 5].

In a previous study, we demonstrated that in primary stimulation cultures of human peripheral blood mononuclear cells (PBM) with the B lymphoblastoid Raji cell line, adriamycin (AM) induced an augmented cytotoxic response under limited conditions [1]. Since mitomycin C (MMC) also has immunomodulating activity [6, 9], the present study was undertaken to investigate the effects of addition of MMC on the development of cytotoxicity of PBM to Raji stimulator cells in culture. The results indicated the augmenting effect of MMC under the limited conditions. Thus, the possible mechanisms involved in the observed augmentation, including MMC-induced effects on interleukins, were investigated.

*Offprint requests to:* Tsuyoshi Akiyoshi

## Materials and methods

*Cell preparation.* Peripheral blood was withdrawn into heparinized syringes from normal donors, and PBM were isolated by a Ficoll-Hypaque density gradient sedimentation. The cells were suspended in RPMI 1640 medium containing 10% pooled human AB serum, supplemented with penicillin 100 units/ml and streptomycin 100 μg/ml (complete medium). To remove the adherent cells, PBM were incubated twice in a plastic dish for 60 min at 37 °C in an atmosphere of 5% $CO_2$ in air. Cells not adhering to the dishes were carefully removed, washed twice, and then resuspended in complete medium. The nonadherent cells contained more than 99% lymphocytes as judged by Giemsa staining and morphology. The adherent cells were obtained by adherence in serum-pretreated plastic dishes. Cells adhering to the dishes were incubated in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS) for 15 min at 4 °C and then mechanically detached. They were washed twice and then resuspended in complete medium. The resulting cell population contained more than 85% monocytes as judged by nonspecific esterase staining.

*Agent.* The MMC (Kyowa Hakko Kogyo Co. Ltd., Tokyo) was dissolved in RPMI 1640 medium immediately before use. An aliquot of the desired concentration of MMC was added to each culture so that the final concentration varied from $2.5 \times 10^{-3}$ to $2.5 \times 10^{-1}$ μg/ml.

*Culture condition.* The basic technique of primary stimulation in culture was that of Potter and Moore [8] with minor modifications. The stimulator or target cells employed were the human B lymphoblastoid Raji cell line. PBM ($1 \times 10^6$) were cultured with Raji stimulator cells in complete medium for 5 days at 37 °C in a humidified atmosphere with 5% $CO_2$ in air. Raji stimulator cells were pretreated with MMC at 50 μg/ml/$10^7$ cells for 60 min, and then washed three times with RPMI 1640 medium. The stimulator cells were added to the mixed cell culture to obtain a responder:stimulator cell ratio of 10:1. In some experiments, PBM were cultured for 24 h bfore antigenic stimulation, during which time they were treated with MMC. In the experiments in which cells were exposed to

MMC for a limited period of time during mixed cell culture, cells were harvested from culture after 24 h and washed three times with RPMI 1640 medium. The cells were then resuspended in the original volume of complete medium and placed back into culture. In every experiment, untreated cultures were also set up as controls. At the end of the 5-day culture period, effector cell populations were recovered and cell viabilities assessed.

*Cytotoxicity assay.* The cytotoxic activity of cells harvested from 5-day culture was determined in a standard 4-h $^{51}Cr$ release assay. Briefly, Raji target cells were radiolabeled with 100 μCi of sodium $^{51}$chromate ($^{51}Cr$) for 1 h at 37 °C. The labeled cells were then washed three times with RPMI 1640 medium. Effector cells harvested from mixed cell culture were placed in each of 4 replicate round-bottomed microculture wells. Then, $1 \times 10^4$ $^{51}Cr$-labeled target cells were added to each well containing effector cells, to 6 wells containing medium alone (to determine spontaneous release), or to 6 wells containing detergent (to determine maximal release). After a 4-h incubation period, the release of $^{51}Cr$ label was measured by collection of cell-free supernatant with Titertek Supernatant Collection System, followed by quantitation in an automated gamma counter. The percentage of specific $^{51}Cr$ release (% cytotoxicity) was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximal release} - \text{spontaneous release}} \times 100$$

*Production of interleukin 1.* The adherent cells ($1 \times 10^6$ cells/ml) were incubated for 24 h at 37° C in the presence or absence of *Escherichia coli* lipopolysaccharide (LPS, 20 μg/ml). The supernatants were obtained by removing the cells by centrifugation. They were filtered through 0.45 mμ Millipore membrane and stored at −20° C until used for interleukin 1 (IL 1) assay.

*Quantification of IL 1.* The IL 1 activity of the supernatants was assayed using the standard thymocyte proliferation assay. Thymocytes were obtained by teasing the thymus glands of 4- to 6-week-old $C_3H/HeJ$ mice. The cells were suspended at $1.5 \times 10^7$ cells/ml in RPMI 1640 medium supplemented with $1 \times 10^{-5}$ *M* 2-mercaptoethanol and 5% FCS. They were cultured for 72 h in the presence of concanavalin A (10 μg/ml) and various dilutions of the samples to be assayed in flat-bottomed microculture plates. Tritiated thymidine (1 μCi/well) was added 6 h before culture termination. The cells were harvested onto glass filtres and counted in a liquid scintillation counter. In every experiment, cultures containing a constant amount of standard IL 1 (recombinant IL 1, Genzyme Co., Boston, Mass USA) were also set up and assayed concomitantly.

## Results

### *Effects of the addition of MMC to culture on the generation of cell-mediated cytotoxicity*

The effects of the addition of MMC at various concentrations on day −1 to day 4 are shown in Fig. 1. MMC was added on the indicated day of culture and removed after exposure for 24 h by washing the cells. All cultures were assayed on day 5 at an effector cell:target cell ratio of 40:1.

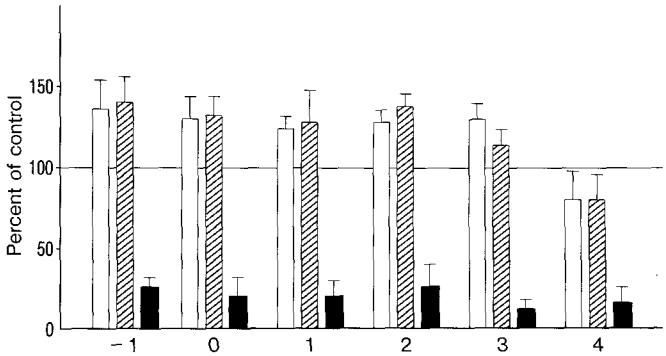


**Fig. 1.** Effects of the addition of mitomycin C (MMC) ($2.5 \times 10^{-3}$ μg/ml □, $2.5 \times 10^{-2}$ μg/ml ▨, $2.5 \times 10^{-1}$ μg/ml □) for 24 h on day −1 to day 4 on the generation of cell-mediated cytotoxicity in primary stimulation culture. The results of 5 experiments are expressed as the percentages of untreated controls normalized to 100. Vertical line, SE. The cytotoxicity was significantly decreased as compared to that of controls when MMC was added on day −1 to day 4 at a concentration of $2.5 \times 10^{-1}$ μg/ml ($P < 0.05$, by Wilcoxon rank test for paired sample). Then, a significant augmentation was observed at concentrations of $2.5 \times 10^{-3}$ μg/ml and $2.5 \times 10^{-2}$ μg/ml on day −1 to day 3 ($P < 0.05$, by Wilcoxon rank test for paired sample)

The cytotoxicity was significantly decreased as compared to that of untreated cultures when MMC was added on day −1 to day 4 at a concentration of $2.5 \times 10^{-1}$ μg/ml. On the other hand, a significant augmentation was observed at concentrations of $2.5 \times 10^{-3}$ μg/ml and $2.5 \times 10^{-2}$ μg/ml on day −1 to day 3.

### *Effect of MMC on adherent and nonadherent cells*

In order to identify the populations of cells affected by MMC, PBM were separated into plastic adherent and nonadherent populations after treatment with MMC

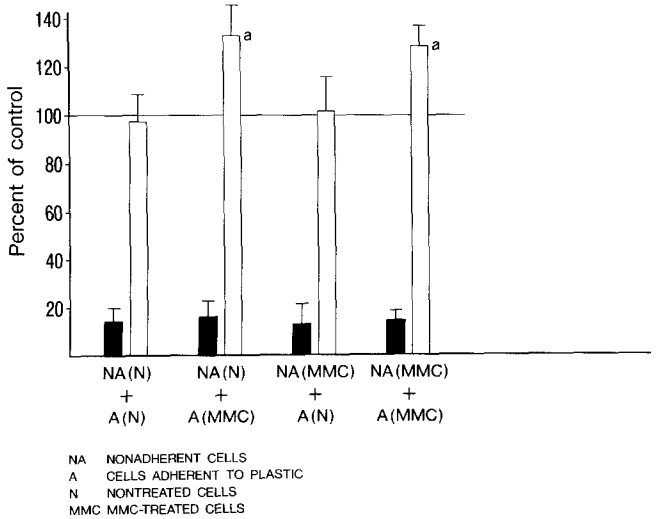


**Fig. 2.** Effects of MMC pretreatment on the cytotoxicity of adherent and nonadherent populations in culture. Peripheral blood mononuclear cells (PBM) were treated with MMC ($2.5 \times 10^{-3}$ μg/ml) or a media control for 24 h (day −1), separated by plastic adherence, and recombined before antigenic stimulation. The results of 5 experiments were expressed as the percentages of untreated controls normalized to 100. The results in cultures of the recombined cells alone were represented when compared to that of untreated PBM ($P < 0.05$, by Wilcoxon rank test for paired sample)

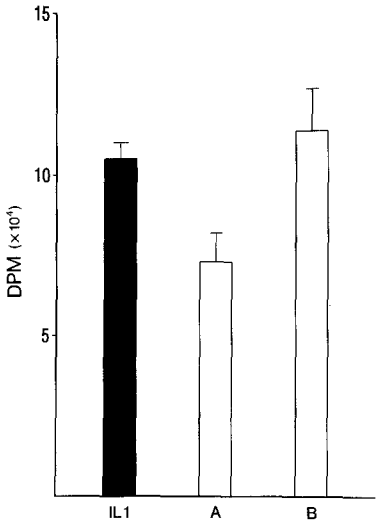


**Fig. 3.** Effects of MMC on interleukin 1 (IL 1) production by adherent cells. PBM obtained from 8 normal donors were treated with MMC ($2.5 \times 10^{-3}$ μg/ml) for 24 h (day −1) and separated by plastic adherence. The adherent cells were cultured with lipopolysaccharide and the supernatant assayed for IL 1 activity. IL 1 production of MMC-treated adherent cells *B* was significantly increased as compared to that of untreated cells *A* ($P < 0.05$, by Wilcoxon rank test for paired sample). Vertical line, SE. Results of the assay containing recombinant IL 1 (1 unit/ml) represented by closed bar (IL 1)

($2.5 \times 10^{-3}$ μg/ml) or a media control for 24 h (day −1). There was no significant difference between the populations of either adherent or nonadherent cells in MMC-treated and untreated cultures. Following cell separation, cultures were reconstituted by combining adherent cells treated with MMC and nonadherent untreated cells and vice versa. Raji stimulator cells were added to the cultures which were then incubated for 5 days. As shown in Fig. 2, the cytotoxic activity was reconstituted to the level of unseparated untreated PBM, when untreated nonadherent cells were separated from adherent cells and then recombined. Similarly, the same augmented cytotoxicity as that of MMC-treated unseparated PBM developed following the recombination of MMC-treated adherent cells and non-adherent cells. However, the cytotoxic activity was not augmented when untreated adherent cells and MMC-treated nonadherent cells were recombined. In contrast, the combination of MMC-treated adherent cells and untreated nonadherent cells resulted in an augmented response. When the recombined cells were cultured with medium alone as controls, there was neither detectable generation of cytotoxic cells nor significant changes in the level of cytotoxicity. The results indicated that augmentation of the cell-mediated cytotoxicity by MMC might be due to a selective effect on the adherent cell fraction.

### *IL 1 production by MMC-treated adherent cells*

To measure the amount of IL 1 produced by MMC-treated adherent cells, PBM were separated into adherent and nonadherent cell fractions after treatment with MMC ($2.5 \times 10^{-3}$ μg/ml) or a media control for 24 h (day −1). MMC treatment did not affect the number of adherent cells. The adherent cells were cultured with LPS for 24 h. Then, the supernatant was collected and assayed for IL 1 activity. As shown in Fig. 3, IL 1 production of MMC-treated adherent cells was significantly increased as compared to that of untreated adherent cells (113,848 ± 11,212 dpm and 72,253 ± 7,256 dpm, respectively).

## Discussion

The present study demonstrated that MMC had immunomodulating activity and, under limited conditions, it could induce augmentation of the cytotoxic activity developed in PBM-Raji cell culture.

In the experimental systems of rats and mice, it was reported that a single injection of MMC into the peritoneal cavity of the animals resulted in enhancement of tumoricidal activity of the peritoneal exudate cells. Then, this activity was found to be associated with the plastic adherent, esterase-positive peritoneal macrophages [6, 9]. In our study, it seemed unlikely that augmentation of the cytotoxicity was due to the direct activation of monocytes, since the addition of MMC to PBM culture alone did not result in augmentation.

In a murine experimental system, Orsini et al. [7] demonstrated that spleen cell populations from mice treated with AM developed a greater cytotoxic response during culture with allogeneic tumor cells than spleen cells from untreated animals. Further, Tomazic et al. [10] reported that addition of AM to culture augmented the development of the cytotoxic response of spleen cells to allogeneic tumor cells in culture. Then, the augmented response was related to effects on the nonadherent fraction of spleen cells, leading to the development of a greater response by T effector cells. However, the results of the present study suggested that the augmented cytotoxicity observed in MMC-treated culture might be related to effects of MMC on the adherent fraction of PBM.

Recently, several investigators reported that the anticancer drugs, especially AM, induced modulation of the soluble mediators of the immune response, i.e., interleukins. In the experimental system where spleen cells from AM-treated mice developed augmented levels of cytotoxic T cell activity in response to alloantigens, an AM-iduced increase in the levels of interleukin 2 (IL 2) activity was observed with isolated cells. The increased levels of IL 2 produced appeared to be a primary mechanism by which AM-induced augmented cytotoxicity occurred [5]. We also demonstrated that PBM obtained from cancer patients produced significantly higher levels of IL2 7 days after AM administration, when the generation of cell-mediated cytotoxicity was significantly augmented [2]. Further, Cohen et al. [3] found that adherent peritoneal exudate cells from mice treated with intraperitoneal AM released an increased amount of a nondialyzable LAF (IL 1)-like factor. Our results showed that adherent cells obtained from MMC-treated PBM produce significantly higher levels of IL 1, when compared to untreated adherent cells.

The interleukins, IL 1 and IL 2, are known to contribute to the regulation of development of cytotoxic T cells, at least in culture. IL 1 which is a monocyte-macrophage product acts in concert with the T helper product, IL 2, to increase cytotoxic T cell activity. Therefore, the augmentation of cell-mediated cytotoxicity in culture by MMC, which appeared to be due to a selective effect on the adherent cells, could be partially explained in relation to the amount of IL 1 produced by the MMC-treated adherent cell population.

## References


1. Arinaga S, Akiyoshi T, Tsuji H (1985) Augmentation of the cell-mediated cytotoxic response induced in mixed cell culture by adriamycin. Jpn J Cancer Res (Gann) 76: 414
2. Arinaga S, Akiyoshi T, Tsuji H (1986) Augmentation of the generation of cell-mediated cytotoxicity after a single dose of adriamycin in cancer patients. Cancer Res 46: 4213
3. Cohen SA, Salazar D, Wicher J (1983) Adriamycin-induced activation of NK activity may initially involve LAF production. Cancer Immunol Immunother 15: 188
4. Ehrke MJ, Tomazic V, Ryoyama K, Cohen SA, Mihich E (1983) Adriamycin induced immunomodulation: Dependence upon time of administration. Int J Immunopharmacol 5: 43
5. Ehrke MJ, Maccubbin D, Ryoyama K, Cohen SA, Mihich E (1986) Correlation between adriamycin-induced augmentation of interleukin 2 production and of cell-mediated cytotoxicity in mice. Cancer Res 46: 54
6. Ogura T, Shindo H, Shinzato O, Namba M, Masuno T, Inoue T, Kishimoto S, Yamamura Y (1982) In vitro tumour cell killing by peritoneal macrophages from mitomycin C-treated rats. Cancer Immunol Immunother 13: 112
7. Orsini F, Pavelic Z, Mihich E (1977) Increased primary cell-mediated immunity in culture subsequent to adriamycin or daunorubicin treatment of spleen donor mice. Cancer Res 37: 1719
8. Potter MR, Moore M (1981) In vitro augmentation of human natural cytotoxicity. Clin Exp Immunol 44: 332
9. Shindo H, Ogura T, Masuno T, Hayashi S, Kishimoto S (1985) Induction of activated macrophages by intraperitoneal injection of mitomycin C in mice. Cancer Immunol Immunother 20: 145
10. Tomazic V, Ehrke MJ, Mihich E (1980) Modulation of the cytotoxic response against allogeneic tumor cells in culture by adriamycin. Cancer Res 40: 2748
11. Tomazic V, Ehrke MJ, Mihich E (1981) Augmentation of the development of immune response of mice against allogeneic tumor cells after adriamycin treatment. Cancer Res 41: 3370


Received August 20, 1986/Accepted December 8, 1986