[CANCER RESEARCH 46, 4213–4216, August 1986]

Augmentation of the Generation of Cell-mediated Cytotoxicity after a Single Dose of Adriamycin in Cancer Patients

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ABSTRACT

The effect of Adriamycin on the generation of cell-mediated cytotoxicity in the mixed cell culture was studied in patients with various carcinomas. Peripheral blood mononuclear cells (PBM) from the patients were cultured with the B-lymphoblastoid cell line Raji in mixed culture, and the induced cytotoxicity was measured by ^{51}Cr release assay. In patients with various carcinomas, the capacity of PBM to be converted to cytotoxic cells was significantly augmented 5, 7, and 10 days after a single dose of 25 mg/sq m i.v., when compared to that of PBM obtained before Adriamycin injection. The peak level of the cytotoxicity observed 7 days after injection was more than 2-fold higher than that before treatment. Although the depletion of adherent cells from PBM either before or after treatment resulted in a decreased cytotoxic response, nonadherent cell fractions as well as unfractionated cells from PBM after drug treatment equally showed an augmented response when compared to that before injection. The distribution of T-cell subsets exhibited a significant increase in the percentage of OKT8 positive cells after administration. Furthermore, PBM obtained after treatment produced significantly higher levels of interleukin 2. The results appear to indicate that the imbalance of T-cell subsets and the increase of interleukin 2 production may be related to the augmenting effect of Adriamycin administration on cytotoxic response in cancer patients.

INTRODUCTION

It has been reported that AM[2] has immunomodulating activity and, depending on conditions, AM could selectively affect particular immune functions, this ultimately resulting in inhibition or augmentation of the immune response.

In a murine experimental system, a single i.p. administration of AM resulted in an increase in cytolytic activity by peritoneal exudate cells of various mouse strains (1). The addition of AM directly to primary stimulation cultures, depending on the time and AM concentrations, augmented the development of the cytolytic response of murine spleen cells to allogeneic tumor cells (2). Then, it was reported that the cell-mediated cytotoxic response of spleen and peritoneal exudate cells from mice immunized with allogeneic tumor was increased when the animals were treated with AM 5 days before immunization (3). Further, spleen cell populations from mice treated with AM were found to develop a greater cell-mediated cytotoxic response during culture with allogeneic tumor cells than spleen cells from untreated animals (4, 5).

In the previous study, we demonstrated that, in primary stimulation cultures of human PBM with the B-lymphoblastoid Raji cell line, AM could induce an augmented cytotoxic response under limited conditions (6). The present study is undertaken to investigate the effect of a low dose of AM to cancer patients on the development of cytotoxic response of PBM to Raji stimulator cells in culture. The results indicate a rather augmenting effect of AM. Thus, the possible mechanisms involved in the observed augmentation are investigated.

Received 11/12/85; revised 4/28/86; accepted 5/6/86.

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[2] The abbreviations used are: AM, Adriamycin; PBM, peripheral blood mononuclear cells; IL 2, interleukin 2; NK, natural killer cell; PHA, phytohemagglutinin.

MATERIALS AND METHODS

Cell Preparation. Thirty-two patients with various carcinomas, including 15 with breast carcinoma, 11 with gastric carcinoma, and 6 with colon carcinoma, were given a single dose of AM (25 mg/m^2 i.v.). Peripheral blood samples were obtained serially from 14 of these patients before and 3, 5, 7, and 10 days after AM administration. In the remaining 18 patients, the blood samples were taken before and 7 days after the drug injection. PBM were isolated by a Ficoll-Conray density gradient sedimentation. The cells were suspended in RPMI 1640 containing 10% pooled human AB serum, supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml) (complete medium). To remove the adherent cells, the cells were suspended at a concentration of 3×10^6/ml in the complete medium and incubated in plastic tissue culture plates (No. 3003; Falcon Plastic, Oxford, CA) for 60 min at 37°C in a humidified 5% CO_2 atmosphere. Cells not adhering to the plates were carefully removed, washed twice, and then resuspended in the complete medium. The preparation contained less than 4% monocytes as judged by esterase staining.

Preparation of Effector Cells. The basic technique of primary stimulation cultures was that of Potter and Moore (7) with minor modifications. The stimulator or target cells used were of the human B-lymphoblastoid cell line Raji. PBM (1×10^6) were cultured with Raji stimulator cells in the complete medium for 5 days at 37°C in a humidified atmosphere with 5% CO_2 in air. Raji stimulator cells were pretreated with mitomycin C at 50 μg/ml/10^7 cells for 60 min and then washed three times with RPMI 1640. The stimulator cells were added to the mixed cell culture to obtain a responder cell:stimulator cell ratio of 10:1. Control cultures containing PBM alone were routinely tested for background cytotoxicity. At the end of the 5-day culture period, effector cell populations were recovered and cell viabilities were assessed according to the trypan blue dye exclusion test.

Cytotoxicity Assay. The cytotoxic activity of cells harvested from 5-day culture or NK activity of freshly obtained PBM was determined in a standard 4-h ^{51}Cr release assay. Raji or K-562 target cells were radiolabeled with 100 μCi sodium [^{51}Cr]chromate for 1 h at 37°C. The labeled cells were then washed three times with complete medium. Effector cells harvested from mixed culture or fresh PBM added to each replicate round bottomed microculture wells. Then, 1×10^4 ^{51}Cr-labeled target cells were added to each well containing effector cells, to 6 wells containing medium alone (to determine spontaneous release), and to 6 wells containing detergent (to determine maximal release). After a 4-h incubation period, the release of ^{51}Cr was measured with a Titertek Supernatant Collection System and quantitated in an automated gamma counter. The percentage of specific ^{51}Cr release (% lysis) was calculated as

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

Production and Quantification of IL 2. PBM were suspended at a concentration of 1×10^6 cells/ml in RPMI 1640 supplemented with 2% human AB serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and PHA (1 μg/ml). After 48 h of culture at 37°C in a 5% CO_2 humidified atmosphere, the supernatant was collected and filtered through a 0.45-nm Millipore membrane. Supernatants were stored at −20°C until used for assay of IL 2 activity. IL 2 dependent cells were

prepared according to the procedure of Tilden and Balch (8). Briefly, PBM obtained from a single volunteer were stimulated with optimal PHA and then recultured in RPMI 1640 supplemented with 25% fetal bovine serum and 25% IL 2 (Boehringer Mannheim GmbH, Mannheim, West Germany) for at least 3 weeks, by which time more than 95% of cultured cells became blastic. Further proliferation of the cells were dependent on addition of exogenous IL 2 but completely unresponsive to PHA. The cultured medium was changed at 3- or 4-day intervals and cell number was adjusted to 10^5/ml. IL 2 activity was quantified by the method of Gillis *et al.* (9). The supernatant tested was processed for 2-fold dilutions and placed in microtiter plates. To these, aliquots (2×10^4 cells) of cultured cells were added. The plates were incubated for 48 h and pulsed with 1 μCi tritiated thymidine for the final 6 h. The cells were harvested onto glass filters and counted in a liquid scintillation counter. In every assay, the uptake of tritiated thymidine by cultured cells in response to the test supernatants was compared to their response to a standard IL 2 preparation (recombinant IL 2; Takeda Chemical Industries, Ltd., Tokyo, Japan) by probit analysis. Results were expressed as units of IL 2 per ml. In some experiments, various dilutions of a monoclonal antibody to IL 2 (IgG1) (Genzyme Co., Boston, MA) were added to the wells at the initiation of the assay to identify the activity. Then, the activity of the supernatant from PBM culture was almost completely abrogated by addition of a 1:2 dilution of the monoclonal anti-IL 2 antibody to the culture.

Monoclonal Antibodies. Monoclonal antibodies used to phenotyping were OKT3, OKT4, and OKT8. Indirect fluorescence was performed by incubating 5×10^5 cells with these antibodies at a dilution of 1:100 for 45 min at 0°C and washing three times in phosphate buffered saline. The cells were then incubated with fluoresceinated goat anti-mouse IgG for additional 30 min at 0°C. After extensive washing, cells were examined directly under a fluorescent microscope, where a minimum of 200 cells were counted.

RESULTS

Effect of AM Administration on the Generation of Cytotoxic Cells or NK Activity. The capacity of PBM from cancer patients to be converted to cytotoxic cells was significantly augmented after a single dose of AM, when compared to that of PBM obtained before AM injection. As shown in Table 1, a significant increase in cytotoxic cell activity was seen 5 days ($P < 0.05$), 7 days ($P < 0.01$), and 10 days ($P < 0.05$) after drug administration. The peak level of the cytotoxicity observed 7 days after AM injection was more than 2-fold higher than that before treatment. In all of these patients, PBM were also cultured with the medium alone to assay the generation of cytotoxic cells as controls. There was neither detectable generation of cytotoxic cells nor significant change in the level of cytotoxicity. NK activity in PBM did not differ significantly from the value before AM administration following the drug treatment.

Table 1 *Changes in the generation of cell-mediated cytotoxicity in the mixed cell culture and in the NK activity following a single dose of AM*

Days after AM administration	Cell-mediated cytotoxicity ($n = 10$)		NK activity ($n = 12$)
	PBM + Raji[a]	PBM alone[b]	
0[c]	14 ± 4[d]	2 ± 1	40 ± 6[e]
3	17 ± 6	4 ± 2	31 ± 5
5	22 ± 5[f]	1 ± 1	44 ± 6
7	32 ± 7[g]	4 ± 1	50 ± 5
10	30 ± 8[f]	4 ± 1	49 ± 8

[a] PBM were cultured with Raji stimulator cells for 5 days and the induced cytotoxicity was measured.
[b] PBM were cultured in the medium alone for 5 days and the induced cytotoxicity was measured.
[c] Before AM administration.
[d] Percentage of cytotoxicity at an effector:target cell ratio of 50:1 (mean ± SE).
[e] Percentage of cytotoxicity at an effector:target cell ratio of 25:1 (mean ± SE).
[f] $P < 0.05$ by Wilcoxon ranks test for paired sample, when compared to the value before AM administration.
[g] $P < 0.01$, as in Footnote *f*.

Effect of the Depletion of Adherent Cells on Cytotoxic Activity. PBM from cancer patients before and 7 days after AM administration were separated to obtain the nonadherent fraction, and the cells were cultured with stimulator Raji cells. Depletion of adherent cells from PBM obtained either before or after AM injection resulted in a decreased cytotoxic response as compared to that of unfractionated cells. However, nonadherent cell fractions as well as unfractionated cells from PBM after drug treatment equally exhibited an augmented response when compared to that before injection (Fig. 1).

T-Lymphocyte Subsets. PBM were obtained from cancer patients before and 7 days after AM administration. The proportion of OKT3 positive cells in PBM after drug administration was slightly increased as compared to that before injection. The distribution of T-cell subsets after injection showed a significant increase in the proportion of OKT8 positive cells compared to that before treatment, whereas there was no significant difference in the percentages of OKT4 positive cells. Then, the OKT4/OKT8 ratio was significantly reduced (Table 2).

IL 2 Production by PBM from Cancer Patients after AM Administration. PBM obtained from patients before and 7 days after AM injection were cultured with PHA. At 48 h of culture, the culture supernatants were collected and assayed for IL 2 activity. The IL 2 activity in the supernatant from culture of PBM obtained 7 days after treatment was increased, when compared to that before injection in 9 of 12 patients. Then, the level of IL 2 produced after injection [0.66 ± 0.07 (SE) unit/ml] was slightly but significantly, higher than that before treatment [0.49 ± 0.06 unit/ml] [$P < 0.05$] [Fig. 2].

DISCUSSION

Several investigators have reported the immunomodulating, especially augmenting, effects of AM on immune responses in experimental system of mice and humans. Santoni *et al.* (1) found that a single i.p. administration of AM resulted in a rapid

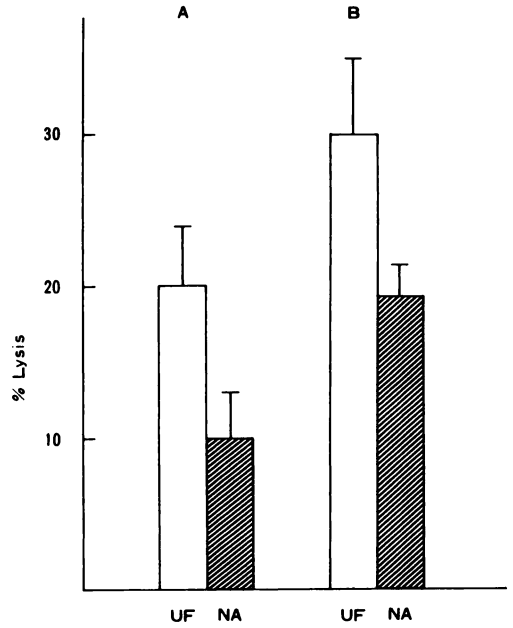

Fig. 1. Effect of the depletion of adherent cells on cytotoxic activity. PBM from 5 patients before (*A*) and 7 days after (*B*) AM administration were separated to obtain the nonadherent fraction (*NA*), and unfractionated cells (*UF*) and nonadherent fraction were cultured with Raji stimulator cells. The induced cytotoxicity was measured at an effector:target cell ratio of 50:1. *Bars*, SE.

Table 2 *Changes in T-lymphocyte subsets following AM administration*

Patient	Before AM administration				7 days after AM administration			
	OKT3	OKT4	OKT8	OKT4/OKT8	OKT3	OKT4	OKT8[a]	OKT4/OKT8[b]
1	72	41	40	1.00	70	31	46	0.67
2	70	41	31	1.32	76	36	50	0.72
3	69	49	22	2.25	68	45	23	2.01
4	48	22	16	1.36	56	27	27	1.00
5	50	29	13	2.31	71	46	25	1.83
6	52	30	12	2.45	63	45	19	2.29
7	89	56	43	1.30	78	41	45	0.90
Mean ± SE	64.3 ± 5.2	38.3 ± 4.2	25.3 ± 4.5	1.71 ± 0.21	68.8 ± 2.6	38.7 ± 2.6	33.6 ± 4.5	1.35 ± 0.23

[a] The proportion of OKT8 positive cells 7 days after AM administration was significantly increased as compared to that before AM administration ($P < 0.05$, by Wilcoxon ranks test for paired sample).

[b] The OKT4/OKT8 ratio was significantly reduced, when compared to that before AM administration ($P < 0.05$, by Wilcoxon ranks test for paired sample).

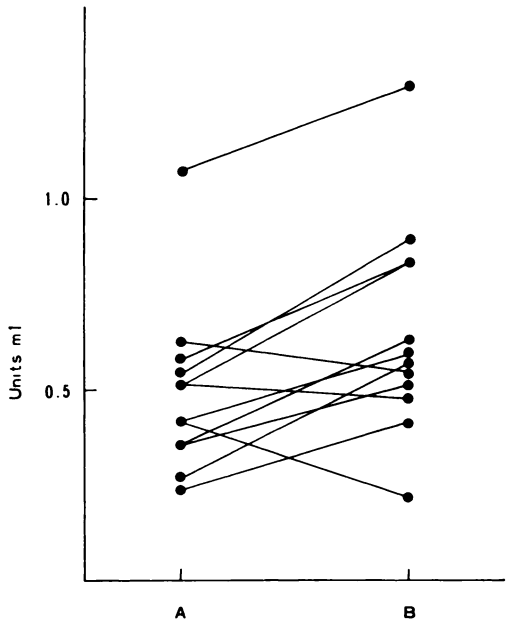

Fig. 2. IL 2 production by PBM from cancer patients after AM administration. PBM obtained from 12 patients before (*A*) and 7 days after (*B*) AM injection were cultured with PHA. At 48 h of culture, the culture supernatants were collected and assayed for IL 2 activity. The level of IL 2 activity was significantly increased after AM treatment ($P < 0.05$, by Wilcoxon ranks test for paired sample).

increase of cytolytic activity by peritoneal exudate cells of various mouse strains, and the effector cells appeared to be NK cells. In humans, Kleinerman *et al.* (10) reported that, utilizing the assay system of spontaneous monocyte-mediated cytotoxicity, treatment of normal PBM with AM *in vitro* enhanced monocyte killing by directly activating the effector cells. However, Haskill (11) suggested that *in vitro* storage of AM by macrophages might be the predominant effector mechanism involved in the AM-activated macrophage phenomenon, in which macrophages harvested from mice given i.p. injections of AM retained cytotoxic activity for 3 or 4 days *in vivo*. In our study, it was unlikely that the augmentation of the cytotoxicity was due to the direct activation of NK cells or monocytes, or *in vivo* drug storage, since NK activity of PBM was not increased and then the cytotoxicity was not detectable when PBM obtained from patients after AM administration were cultured in the medium alone.

In a murine experimental system, Tomazic *et al.* (2) reported that the addition of AM directly to primary stimulation culture augmented the development of the cytotoxic response of spleen cells to allogeneic tumor cells. 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. Further, Orsini *et al.* (4) demonstrated that spleen cell populations from mice treated with AM developed a greater cytotoxic response during culture with allogeneic tumor cells than spleen from untreated animals. Then, the cells responsible for the development of this increased response were concentrated in the nylon wool adherent fraction, non-T-cells. Their further studies (12, 13) showed that AM induced selective modifications in both the nonadherent, nonphagocytic monocyte-macrophage precursor and the adherent T-regulatory cell and that, as a consequence, augmented levels of cell mediated cytotoxicity could develop. In the present study, nonadherent fractions as well as unfractionated cells from PBM after AM injection equally exhibited an augmented response, although the depletion of adherent cells either before or after treatment resulted in a decreased response. Therefore, the nonadherent cell fraction might be mainly responsible for the augmentation of the response. However, it seemed probable that, as shown in murine experiments, immature cells in nonadherent fraction developed into functionally mature macrophages during culture and the cells contributed accessory function (12, 13).

The effects of anticancer drugs on human T-lymphocyte subsets have been reported by several authors. Berd *et al.* (14) showed that, following administration of cyclophosphamide, the number of T-lymphocytes decreased without selective depletion of helper/inducer or suppressor/cytotoxic T-cells. However, Ben-Efrain *et al.* (15) demonstrated that *in vitro* treatment of human PBM with melphalan significantly decreased both the percentage of total T-lymphocytes and the percentage of OKT4 positive cells, whereas the percentage of OKT8 positive cells remained unchanged. In contrast, Lauria *et al.* (16) found that patients treated for Hodgkin's disease, disease free and not receiving therapy for over 5 years, showed a significant imbalance in T-cell subsets, with a significant increase in OKT8 positive cells and near normal OKT4 positive cells. In our study, the distribution of T-cell subsets in PBM from cancer patients after AM administration also showed a significant increase of OKT8 positive cells, while no significant difference was observed in the OKT4 positive cell populations. The interpretation of this T-cell subset imbalance requires further investigation. However, the possibility that it may be related to the reaction towards the disease, thus indicating a state of immunological surveillance, is suggestive, since the OKT8 positive cell population is composed of both suppressor and cytotoxic T-cells, and the capacity to produce cytotoxic cells is shown to be augmented at this period in this study.

IL 2 is a cytokine that plays an important role in the development of cytotoxic cell response. AM was shown to diminish IL 2 production of human PBM after stimulation with PHA *in vitro* (17). In the experimental system in which spleen cells from AM treated mice could develop augmented levels of cytotoxic T-cell activity in response to alloantigens, however, AM induced increase in the levels of IL 2 activity was observed

with isolated cells. Then, the increased levels of IL 2 produced appeared to be a primary mechanism by which AM induced augmented cell mediated cytotoxicity occurred (18). Our results also showed that the level of IL 2 produced in the supernatant from culture of PBM obtained after AM treatment was slightly, but significantly, higher than that before injection. Therefore, the modification in IL 2 production appeared to play a role in AM induced augmentation of cytotoxic activity in patients.

It was thought that IL 2 was mainly released by helper/inducer T-cells identified by OKT4 (19, 20). However, Meuer *et al.* (21) reported that both OKT4 and OKT8 positive cells produced IL 2 by stimulation with mitogens. The present results showed that OKT4 positive cell population in PBM after treatment was not significantly altered, with a significant increase of the percentage of OKT8 positive cells.

The results presented here suggest that the imbalance in T-cell subsets and the increase of IL 2 production in PBM may be involved in the phenomenon, in which the capacity of PBM from cancer patients to generate cytotoxic cells was significantly augmented after a single dose of AM. Our findings appear to indicate an augmenting effect of AM on immune response in cancer patients and, further, to allow the combination of optimal AM administration with immunotherapy in the treatment of cancer patients.

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