Effects of Adriamycin and Cyclophosphamide Treatment on Induction of Macrophage Cytotoxic Function in Mice

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ABSTRACT

The effects of i.p. and s.c. Adriamycin and cyclophosphamide treatment of BALB/c × DBA/2 F1 mice were studied alone and in combination with immunotherapeutic agents, pyran copolymer and Bacillus Calmette-Guérin, on macrophage cytotoxic ability. As assessed by direct viable cell counts of MBL-2 leukemia cells, both Adriamycin and cyclophosphamide produced growth-inhibitory macrophages. This function after s.c. cytostatic treatment peaked at Day 1 and decreased progressively, attaining normal control values by Day 8. When adjuvants, such as pyran and B. Calmette-Guérin, were administered i.p. simultaneously with s.c. Adriamycin or cyclophosphamide, adjuvant-induced cytotoxic function was not markedly affected. A better knowledge of the influence of cytostatic agents alone or combined with immunoadjuvants on macrophage cytotoxic ability may be useful in designing more effective chemoinmunotherapy protocols.

INTRODUCTION

ADR and CY are currently used in combination chemotherapy in a wide range of human neoplasms (1, 8, 9, 13). Therapeutic synergism between these cytostatics was established for a large number of transplantable animal tumors (2, 5, 28), due to a dramatic inhibition of tumor cell DNA synthesis (24). Both ADR and CY have been shown to be suppressive on humoral and cellular immune responses under certain treatment conditions (6, 17, 26), while under other conditions stimulatory effects have been observed (3, 7, 25, 27).

Mantovani et al. (11), in their study on the effect of ADR on the induction and expression of macrophage cytotoxicity, established that in vivo ADR treatment impaired the cytotoxicity of Corynebacterium parvum-activated splenic macrophages against leukemia cells in vitro, when assessed by means of [125I]Iododeoxyuridine uptake. The same results were obtained when peritoneal macrophages were used as effector cells (10). In contrast, Schultz et al. (23) recently reported that peritoneal macrophages from CY-treated mice were transiently cytostatic for MBL-2 leukemia target cells. The effects of cytostatics on macrophage functional activity have not been sufficiently characterized. This is especially important since immunoadjuvants, which presumably work in part by macrophage activation (e.g., BCG, C. parvum, and glucan), have been used empirically in cancer treatment in combination with cytostatic agents without thorough knowledge of their compatibility. Results presented in this report describe the effects of ADR and CY when used alone or in combination with the macrophage activators pyran copolymer and BCG on macrophage cytotoxic activity and on the number of macrophages residing in the peritoneal cavity.

MATERIALS AND METHODS

Mice. Male BALB/c × DBA/2 F1, (hereafter called CD2F1; H-2b) mice were supplied by the Mammalian Genetics and Animal Production Section of NIH, Bethesda, Md. All animals were 6 to 8 weeks old and weighed approximately 25 g when used for experimentation. The mice were housed in plastic cages and given Purina laboratory chow and tap water ad libitum.

Peritoneal Macrophages. Peritoneal fluids were harvested from CD2F1 mice after i.p. injection of 5 ml of serum-free Roswell Park Memorial Institute Medium 1640, containing 2 units of heparin per ml. Within 5 to 10 min after injection, the animals were sacrificed by cervical dislocation, and the peritoneal fluid was withdrawn by syringe aspiration. Peritoneal cells from 5 to 8 mice in each group were pooled, washed once in 20 ml RPMI-FCS, and purified by adherence as described previously (22). Approximately 4 × 10^6 macrophages were seeded into 16-mm wells on Cluster 24 tissue culture plates (Costar, Cambridge, Mass.) and incubated for 90 min; macrophage monolayers were washed thoroughly with jets of medium before use in experiments. By this means, it was estimated that >95% of the cells had morphological characteristics of macrophages.

Target Cells. MBL-2 lymphoblastoid leukemia cells were maintained as suspension cultures in RPMI-FCS. Viability of the cells was determined by the trypan blue exclusion test.

Drugs. ADR and CY were kindly supplied by the Drug Research and Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Both drugs were dissolved in PBS. Pyran copolymer (NSC 46015) was obtained from Dr. David Breslow of the Hercules Research Center, Wilmington, Del. Pyran was dissolved in PBS, and the pH was adjusted to 7.2 by addition of 0.1 N sodium hydroxide. BCG was obtained from Dr. S. D. Chaparas, Bureau of Biologics, Food and Drug Administration, Rockville, Md. BCG was suspended in pyrogen-free PBS. Purified mouse fibroblast interferon (specfic activity, 2 × 10^7 units/mg of protein) was provided by Dr. K. Paucker, Medical College of Pennsylvania, Philadelphia, Pa.

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The interferon was dissolved in PBS and injected i.p. ADR and CY were administered s.c. and i.p. at doses calculated to result in LD_{10}, 0.5 LD_{10}, and 0.25 LD_{10}, as established in CD2F1 mice by Southern Research Institute, Birmingham, Ala. The LD_{10} is close to the maximum tolerated doses in chemotherapeutic experiments. Pyran, BCG, and PBS (used as placebo) were administered i.p., and all drugs were injected into mice in a volume equal to 1% of their body weight.

**Macrophage Cytotoxicity Assay.** The assay for measuring macrophage-mediated cytotoxicity has been described previously (20, 23). Approximately 4 x 10^5 macrophages in 16-mm wells were overlaid with 4 x 10^4 MBL-2 cells contained in 2 ml of RPMI-FCS. All cultures were maintained in a humidified, 5% CO_2-in-air incubator at 37°, and cytotoxicity was assessed at 48 hr on the basis of viable cell counts in a hemocytometer. Triplicate cultures were maintained for each group; the mean cell count and S.E. were calculated. Under these conditions, peritoneal macrophages from PBS-treated normal CD2F1, mice did not affect the growth of MBL-2 target cells, as measured both by viable cell number and by DNA synthesis of the leukemia cells. The ratio of macrophages to target cells was approximately 10:1 at the beginning of each experiment. The percentage of growth inhibition of MBL-2 cells due to drug-mediated macrophage activation was calculated by comparison to that of MBL-2 cells grown in the presence of macrophages from PBS-treated animals. Each experiment was performed 3 times, and similar results were obtained.

**RESULTS**

**Effects of Dose and Route of Administration of ADR and CY on Macrophage-mediated Cytotoxicity.** We have established previously the MBL-2 cell growth inhibition assay as a sensitive system to monitor the cytotoxic activity of activated macrophages (20, 23). We have routinely used mouse L-cell interferon as a positive control due to its known ability to evoke the appearance of activated macrophages (18, 19). Although macrophages from uninfected, control animals did not significantly alter MBL-2 cell proliferation, macrophages harvested 1 day after interferon treatment (1 x 10^4 units/mouse i.p.) inhibited MBL-2 cell proliferation by approximately 60 to 70%. A typical experiment is shown in Table 1.

Peritoneal macrophages harvested 1 day after i.p. ADR administration inhibited MBL-2 cell proliferation; the highest value of growth inhibition of MBL-2 cells was obtained with the highest dose of ADR (Chart 1). Macrophages from mice treated i.p. with CY also inhibited MBL-2 cell growth, although the highest inhibition was observed with the lowest dose of CY tested (Chart 1). To exclude direct effects of i.p. drug treatment on peritoneal macrophages, macrophage reactivity was also tested following s.c. administration of cytokistatic. The kinetics of macrophage cytotoxicity with s.c. ADR treatment was similar to that observed in i.p.-treated mice (Chart 2). However, macrophages originating from s.c. CY-treated mice were more cytotoxic against MBL-2 cells after administration of the higher (LD_{10}) dose than after the administration of the 0.25 LD_{10} observed with i.p.-treated mice (compare Chart 1 to Chart 2). Both i.p.- and s.c.-administered cytokistatics did not significantly affect the cell number of resident peritoneal macrophages at Day 1 after treatment.

**Chronological Appearance of Macrophage-mediated Cytoxicity.** In 3 separate experiments, macrophages from 8 normal CD2F1 mice from each group were harvested and pooled 6 consecutive days after s.c. treatment with ADR and CY and tested for their cytotoxic ability. ADR and CY administered at LD_{10} doses (10 and 300 mg/kg, respectively) produced growth-inhibitory macrophages with maximal activity at Day 1 after treatment. These effects fell off gradually, reaching normal control values by Day 6 (Chart 3). The yield of recoverable peritoneal macrophages was similarly tested at various times after drug treatment. No significant changes in macrophage number were observed compared to these in PBS-treated controls (Table 2).

**Effects of ADR and CY on Macrophage-mediated Cytotoxicity by Pyran Copolymer and BCG.** To determine whether ADR and CY affect the ability of pyran or BCG adjuvants to...
induce growth-inhibitory macrophages, the adjuvants or PBS placebo were administered i.p. simultaneously with s.c. injection of ADR (10 mg/kg) or CY (300 mg/kg) 6 days prior to harvesting peritoneal macrophages. By the sixth day after ADR or CY treatment, the functional activity of macrophages to inhibit MBL-2 cell proliferation had almost completely declined (Chart 3). In contrast, macrophages from mice treated simultaneously with cytostatics (ADR or CY) and pyran or BCG showed the same cytotoxic activity as those originating from mice given adjuvants alone (Chart 4).

**DISCUSSION**

Since ADR and CY are being used in conjunction with immunotherapeutic agents in the treatment of human neoplasms, the present study was undertaken to determine the effect of these cytostatics when used alone or in combination with pyran or BCG on macrophage tumoricidal function. The results show that macrophages, harvested within 1-4 days from mice given i.p. or s.c. injections of ADR or CY, manifest increased cytotoxicity for MBL-2 leukemia cells in vitro.

In showing that ADR itself possesses a macroleage-stimulatory effect, as assessed by direct count of viable leukemia target cells, our findings are in contrast with those of Mantovani et al. (10, 11). While Mantovani et al. found that ADR abrogated the cytotoxic activities of C. parvum-activated splenic (11) and peritoneal (10) macrophages, we found that the functional activity of pyran- or BCG-activated macrophages was not significantly impaired. In addition, Mantovani et al. described that in their cytotoxicity assay (using [125I]iododeoxyuridine uptake by TLX9 and SL2 target cells) no enhanced activity with ADR was noted on the function of macrophages in the absence of C. parvum. These differences may be related to the sensitivity and technical problems encountered in their cytotoxicity assay as noted by the authors themselves (10). It should be noted that our own experience has shown that the number of leukemia cells in the presence of normal macrophages does not vary markedly from that of leukemia cells grown alone, as measured both by direct viable cell counts and by [3H]thymidine incorporation.

The cause of the increased transient activity of macrophages following administration of maximum tolerated doses of ADR or CY is not presently known. However, the early response of macrophages to exogenously added interferon (Chart 1; Table 1) is similar to that seen with ADR and CY. It is possible that these 2 cytostatics are lytic to lymphocytes (7, 10), which could allow for the release of preformed mediators capable of activating macrophages. A direct interaction of macrophages with ADR is possible in the enhanced macrophage-mediated cytotoxicity, since Facchinetti et al. (4) showed that macrophages exposed to ADR in vitro take up the drug progressively. It is possible that macrophages collected from the in vivo condition could merely transport the cytostatic agents into the culture medium and thereby inhibit MBL-2 cell growth. However, this mechanism appears unlikely, due to the fact that conditioned media from ADR- or CY-treated macrophages did not suppress MBL-2 cell growth.3

The ability of T-lymphocytes from ADR- or CY-treated mice to produce macrophage-activating factor was not severely compromised, since BCG, a lymphocyte-dependent activator of macrophages (15), was able to render macrophages cytotoxic in cytostatic-treated mice (Chart 4). The number of peritoneal macrophages was not significantly altered after ADR treatment as has been noted previously by Mantovani (10).

Since tumor burden was reported not to markedly affect the tumoricidal effect of in vivo BCG-activated macrophages (12), the observation that ADR and CY did not affect the ability of pyran copolymer and BCG to induce cytotoxic macrophages is of interest. The transient immunosuppressive effect of ADR or CY would not compromise their ability to act as both direct cytostatic agents and macrophage-activating agents. Further studies are in process to determine the compatibility of other clinically active cytostatic drugs combined with immunotherapeutic agents on maintenance and expression of activated macrophage functional activity.

3 Unpublished observation.
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REFERENCES

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