Adriamycin-activated Macrophages as Tumor Growth Inhibitors

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ABSTRACT

Flow cytometry has been used to study the influence of Adriamycin- and Corynebacterium parvum-elicited cytostatic macrophages on the cell cycle of HeLa cells. Macrophages harvested from the peritoneal cavity of mice given i.p. injections of Adriamycin retain cytostatic activity for 3 to 4 days in vivo. Many of these macrophages fluoresced with the red emission spectra characteristic of Adriamycin. The decline in the presence of macrophages exhibiting red fluorescent cytoplasmic particles paralleled the loss in cytostatic activity.

Drug-induced cytostatic macrophages exhibited at least as high a level of growth inhibition as did C. parvum-activated cells. In contrast to the apparent G₀ block of HeLa cells which resulted from interaction with C. parvum-activated macrophages, Adriamycin-stimulated macrophages inhibited growth in the G₂-M phase characteristic of the free drug. Multiple freeze-thaw cycles removed all activity from C. parvum macrophages but had no influence on the Adriamycin-elicited effector cell activity. The data suggest that in vivo drug storage by macrophages may represent an important step in tumor cell destruction by Adriamycin.

INTRODUCTION

Adriamycin is a potent antitumor agent for a variety of clinical and experimental tumors. There has been considerable interest in the influence that such a drug may have on the immune system (2, 7, 10, 11, 15, 16) and in particular on the activation of macrophages by biological modifiers such as Corynebacterium parvum and Bacillus Calmette-Guérin (7, 8, 12). It has generally been assumed that most anticancer drugs manifest a favorable therapeutic value in spite of their immunosuppressive properties. It has been reported recently that macrophages can store drugs such as Adriamycin in vivo and may remain cytostatic for tumor cells for up to 5 days following drug administration (2, 15). Thus, it is possible that certain drugs may additionally influence tumor survival indirectly via macrophages.

These data raise the important question of whether Adriamycin is capable of activating macrophages in a similar fashion to agents such as C. parvum or the cytostatic mechanism is mediated via drug storage in the primary lysozomal granules of the macrophage (2).

In the present study, we have investigated the effect of C. parvum- and Adriamycin-activated macrophages on HeLa cell progression through the cell cycle. The results indicate distinct mechanisms of cytostasis for the 2 effector cells. The results also support the contention that, in a manner similar to free drug, Adriamycin-activated macrophages are growth inhibitory to tumor cells predominantly in the G₂ phase of the cell cycle.

MATERIALS AND METHODS

Animals

Eight to 12-week-old female C57BL/6 × A F₀ (hereafter called B6AFᵢ) mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Introduction of Activated Macrophages

C. parvum Stimulation. Mice were given i.p. injections of 0.2 ml C. parvum (7 mg, dry weight, bacteria per ml) kindly donated by Dr. Richard L. Tuttle from Burroughs Wellcome, Research Triangle Park, N. C. C. parvum were harvested 5 days later. No attempt was made to maintain endotoxin-free conditions, and materials were not screened for the presence of endotoxins.

Adriamycin Stimulation. B6AFᵢ mice were given injections of graded doses of freshly prepared drug in 0.9% NaCl solution. Preliminary experiments indicated a similar dose-response relationship to that published by Stoychov et al. (15). Therefore, a dose of 250 µg/mouse was used routinely.

Tissue Culture. All assays were carried out in α-modified minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum (K. C. Biological, Inc., Lenexa, Kans.) and 20 µg gentamicin sulfate (Sigma Chemical Co., St. Louis, Mo.) per ml.

Target Cells. HeLa 71 cells provided by Dr. Barry Goz, Department of Pharmacology, University of North Carolina, Chapel Hill, N. C., were used in all assays.

Preparation of Macrophage Effector Cells. Peritoneal exudate cells were harvested by flushing the peritoneal cavity with 10 ml α-modified minimal essential medium. Cytocentrifuge slides were prepared and stained with Diff-Quick stain (Harleco, Gibbstown, N. J.) and 90% of the cells were macrophages. We have previously shown that this reagent is specific for macrophages (4). Peritoneal cells were incubated in a 1:100 dilution of the IgG fraction of the absorbed antiserum, washed, and stained with fluorescein-conjugated goat anti-rabbit IgG (N. L. Cappel Laboratories, Inc., Cochranville, Pa.). This reagent does not stain neutrophils. In addition, visual examination of the cells indicated Adriamycin was not present in the cytoplasm of cells other than macrophages.

Identification of Macrophages. Macrophages were identified with the aid of a rabbit antiserum produced against mature mouse bone marrow macrophage colony-derived cells. We have previously shown that this reagent is specific for macrophages (4). Peritoneal cells were incubated in a 1:100 dilution of the IgG fraction of the absorbed antiserum, washed, and stained with fluorescein-conjugated goat anti-rabbit IgG (N. L. Cappel Laboratories, Inc., Cochranville, Pa.). This reagent does not stain neutrophils. In addition, visual examination of the cells indicated Adriamycin was not present in the cytoplasm of cells other than macrophages.

Instrumentation. An Ortho ICP-22 flow cytometer was used for all measurements. Single-parameter DNA profiles were stored directly in the storage facility of the ICP-22 and, if needed, subtracted using the SUB mode. For 2-parameter analysis, a Radio Shack (Radio Shack Corp., Fort Worth, Texas) TRS-80 Model 1 microcomputer was inter-
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Characterization of the Effector Cell Responsible for Drug Retention. Facchinetti et al. (2) have extensively studied the localization patterns of a variety of drugs including Adriamycin into macrophages. Shortly after in vitro exposure, Adriamycin, characterized by its red fluorescence, was seen to accumulate in cytoplasmic inclusions. We observed a similar pattern following in vivo administration [microscopic examination (data not shown)]. In order to examine this in more quantitative terms, peritoneal cells from animals exposed in vivo to Adriamycin for 4 hr were analyzed by flow cytometry using the red fluorescence from the Adriamycin-containing cells as one parameter and indirect immunofluorescence with an antimacrophage reagent as the second parameter. The cytogram indicated that several populations of the cells contained Adriamycin. One, which gave intense red fluorescence on many of the cells, was reactive with the antimacrophage stain (Chart 2, top) (con-
firmed by visual observation). The remaining cells including neutrophils had only the pale background red fluorescence resulting from the expected DNA staining of the nucleus by Adriamycin (Chart 2, bottom). The data thus suggest that the majority of the Adriamycin present in the peritoneal cells used for cytostasis studies was localized in the cytoplasmic granules of macrophages.

Comparison of Cytostatic Capacity of Adriamycin and C. parvum-activated Macrophages. Dose-response relationships were obtained for growth inhibition assessed by both a postlabeling technique ( incorporation) and quantitative flow cytometry. Growth inhibition was marked even at the 5:1 ratio with the Adriamycin macrophage population (flow cytometric determination) (Chart 3, top). Although the degree of cytostasis by both Adriamycin- and C. parvum-activated macrophages was not as pronounced with the postlabeling assay, the data confirms the growth-inhibitory nature of Adriamycin-activated macrophages reported by Stoychkov et al. (15) using a similar assay. Cytotoxicity was more pronounced when the C. parvum macrophage influence on cell survival was determined (Chart 3, bottom), indicating that most of the Adriamycin macrophage influence was probably growth inhibitory rather than cytolytic.

Cell Cycle Specificity of the Adriamycin Macrophage Effect. Adriamycin has several influences on tumor cell progression through the cell cycle, the most pronounced one being arrest in the G2 phase (3). Flow cytometric analysis using quantitative propidium iodide staining for DNA can distinguish target and effector cells (Chart 4). Using this type of analysis, we have been able to demonstrate that, in contrast to C. parvum macrophages which block HeLa cells in the G1-S phase of the cell cycle, Adriamycin macrophages appeared to have a different influence more restricted to the G2-M part of the cycle (Chart 5).

A dose-response relationship between the proportion of HeLa cells in the G2-M growth fraction and the ratio of normal, C. parvum-activated, or Adriamycin-stimulated macrophages was determined (Chart 6). The data clearly indicate the progressive blocking of cells in the G2-M phase resulting from Adriamycin macrophage treatment in contrast to what was observed with C. parvum-activated macrophages.

Direct Evidence for a Cell Factor Rather Than Cell Contact. Stoychkov et al. (15) have reported that overnight supernatants of Adriamycin-treated macrophages are not growth inhibitory, thus suggesting that direct contact with the macrophage is essential. Although we too have failed to obtain a supernatant which was growth inhibitory, we did observe that drug-containing macrophages were no longer detectable after 24 hr in vitro and that free drug lost activity at 37°C overnight (data not shown). Therefore, we carried out a series of experiments to investigate whether or not cell contact was required. Duplicate assay trays containing normal, C. parvum-activated, and Adriamycin-activated macrophages were prepared; one was frozen and thawed 4 times until only cell debris remained. Both plates then received target cells, and postlabeling growth inhibition assays were carried out. The results clearly indicated that, unlike C. parvum macrophages, disrupted drug-elicited macrophages were active (Table 1), suggesting that cell contact was unnecessary for cytostasis.

DISCUSSION

There has been considerable interest in the influence that chemotherapeutic agents such as Adriamycin can have on the immune system (2, 7, 11, 12, 15, 16). This results from the apparently antagonistic influences that many drugs may have on the tumor-bearing host: tumor cell destruction concomitant
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Chart 5. DNA histograms for HeLa cells cocultured with normal (N Mφ), C. parvum-stimulated (Cp Mφ), or Adriamycin-stimulated (Adr Mφ) macrophages. The histograms demonstrate that the C. parvum macrophages inhibited migration of tumor cells out of S phase into G2 + M phases of the cell cycle, whereas Adriamycin macrophages caused a piling up of cells in the G2 + M phases. A 10:1 effector:target cell ratio was used (24-hr assay). The macrophage peak, Channel 15, has been gated out as HeLa 71 cells are markedly aneuploid (1.8 times normal). The histogram for HeLa cells alone was similar to that with normal macrophages. The identity of HeLa cell in the presence of effector cells has been confirmed by 2-parameter analysis.2

with immune suppression. Several reports have detailed the influence that drugs have on macrophage activation for tumor growth inhibition (11, 15). With one exception, these studies have not investigated whether or not macrophages exposed to drug might themselves be tumoricidal (10,15). This is important as there are numerous reports that the presence of a mononuclear cell infiltrate (including macrophages) is associated with a more favorable response to therapy (5, 6). In addition, there are several reports of animal tumor systems which depend on intratumor immunity for a successful chemotherapy response (12-14). Thus, in some cases, drugs may work more effectively as a result of the in situ inflammatory response.

Stoychkov et al. (15) recently reported the exciting obser-

Table 1

<table>
<thead>
<tr>
<th>% of growth inhibitiona</th>
<th>20:1</th>
<th>10:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plate 1 (control)</td>
<td>Normal</td>
<td>18 ± 15b</td>
</tr>
<tr>
<td></td>
<td>Adriamycin</td>
<td>85 ± 4</td>
</tr>
<tr>
<td></td>
<td>C. parvum</td>
<td>94 ± 2</td>
</tr>
<tr>
<td>Plate 2c (freeze-thawed)</td>
<td>Normal</td>
<td>4 ± 9</td>
</tr>
<tr>
<td></td>
<td>Adriamycin</td>
<td>83 ± 9</td>
</tr>
<tr>
<td></td>
<td>C. parvum</td>
<td>4 ± 6</td>
</tr>
</tbody>
</table>

a Postlabeling assay; 20:1 and 10:1, effector:target cell ratios.
b Mean ± S.D. of 4 experiments.
c Prior to addition of target cells, plate was subjected to 4 cycles of freezing (-20°) and thawing (37°). Adriamycin macrophages (24 hr postinjection: 250 μg i.p.); C. parvum macrophages (5 days postinjection; 700 units C. parvum i.p.).

vation that both Adriamycin and cyclophosphamide administered i.p. or s.c. induced growth-inhibitory macrophages which survived in vivo for 5 to 6 days. The degree of macrophage stimulation was comparable with that obtained following interferon treatment. The authors felt that, although drug was retained by the peritoneal macrophages, a direct influence of drug released from macrophages into the medium was unlikely as culture supernatants were inactive.

We have investigated to see if Adriamycin is stored or activates macrophages by using quantitative flow cytometry to analyze both the effector cells retaining the highly fluorescent drug (2) and the cell cycle specificity of the resulting effector-target cell interaction. We have obtained data that indicate that macrophages are the predominant adherent cell to retain drug and that Adriamycin macrophages had a strong growth-inhibitory influence on HeLa cells quantitatively comparable to that resulting from C. parvum-activated macrophages but qualitatively distinct.

Several groups have reported that activated macrophages may have a specific cell cycle effect on tumor cell progression (8, 9). We have observed that C. parvum-stimulated macrophages inhibit HeLa cell growth at or near the G1-S interphase.2 The present data indicate that Adriamycin macrophages cause a progressive accumulation of target cells at a different part of the cell cycle in the G2-M growth fraction. This fact, taken together with the storage of drug by the macrophages, would appear to supply strong evidence that drug retention may be the predominant effector mechanism involved in the Adriamycin-activated macrophage phenomenon. This is further sub-

Chart 6. Dose-response relationship between Adriamycin (Adr Mφ), normal (N Mφ), and C. parvum macrophages (Cp Mφ) (40-hr assay). The various percentages of HeLa cells in the G2 + M phase of the growth fraction were quantitated in individual wells of cytostasis assays. Points, average of triplicate wells. HeLa cells cultured in the absence of effector cells were 18% in the combined G2 + M growth fraction.
stantiated by our observation that freezing and thawing the macrophages does not destroy activity unlike that due to C. parvum-activated macrophages.

Further studies using drugs with distinct cell cycle specificities and target cell lines selected for drug resistance are underway to investigate whether or not the Adriamycin observations are unique. Storage of a highly active drug in macrophages for periods of hr or days in vivo could have important antitumor influences. This is an especially exciting possibility for the direct intracavitary administration of chemotherapy for patients with abdominal or pleural malignant effusions which frequently contain significant numbers of macrophages.

REFERENCES

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