Activation of Tumor-cytostatic Macrophages with the Antitumor Agent 9,10-Anthracenedicarboxaldehyde Bis[(4,5-dihydro-1H-imidazole-2-yl)hydrazone] Dihydrochloride (Bisantrene)

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ABSTRACT

An investigation was carried out to determine the potential for activating tumor-inhibitory macrophages with the cytotoxic antitumor agent, bisantrene. Macrophages prepared from peritoneal exudates of mice treated i.p. with bisantrene were extremely active in inhibiting the growth of tumor cells. The minimal effective in vivo dose of this drug appeared to be 25 mg/kg, with peak activation being achieved at doses of 50 to 100 mg/kg. Effector cells became detectable 2 days after treatment and persisted for at least 4 weeks. Incubation of effector and target cells for 48 hr seemed necessary to achieve the maximum inhibitory effect.

Treatment with carrageenan in vitro and in vivo abolished tumor cytostasis, whereas treatment with anti-T-cell antibody plus complement had no effect, suggesting that macrophages rather than T-lymphocytes were responsible for the observed results. Culture supernatants of activated macrophages were found to have antiproliferative effects on tumor cells, indicating that a cytosstatic factor(s) was produced by these macrophages. Hydrogen peroxide and neutral proteases apparently did not function as cytosstatic mediators since activated macrophages were resistant to treatment with catalase, N-α-p-tosyl-L-lysine chloromethyl ketone, and aprotinin. The present findings suggest that, in addition to direct toxicity to tumor cells, bisantrene may act as a macrophage-activating immunopotentiator. This observation may be of potential clinical usefulness in the design of immunochemothepapeutic trials for certain types of cancer.

INTRODUCTION

Macrophages have been considered important to the host defenses controlling the development and spread of neoplasms (13). Such a role has been supported by experimental evidence demonstrating that macrophages are capable of destroying tumor targets in vitro. Furthermore, macrophage-mediated tumor destruction can be augmented by a variety of substances, including several chemotherapeutic agents, e.g., cyclophosphamide (25), Adriamycin (14, 25), and mitomycin C (17).

Bisantrene is a novel antitumor agent active against a number of experimental tumors, including P388 leukemia, L1210 leukemia, Lieberman plasma cell tumor, B16 melanoma, colon tumor 26, and Riddway osteogenic sarcoma (6). The drug is effective over a dose range of 1.56 to 150 mg/kg depending upon the frequency, route, and schedule of the treatment and the tumor model used. Animal toxicity studies in beagles and cynomolgus monkeys showed that bisantrene caused a dose-related but reversible bone marrow hypoplasia which resulted in leukopenia and anemia. No cardiotoxicity was observed in dogs receiving the compound at 64 or 128 mg/sq m every 3 weeks for 31 weeks (24). Bisantrene is currently in Phase II clinical trial (28).

In this study, we have investigated the potential immunopotentiating effects of bisantrene as a result of an observation demonstrating that the compound given prophylactically protected mice from lethal bacterial challenge despite the fact that it has no in vitro antibacterial activity. The present findings indicate that bisantrene activates macrophages inhibitory to the growth of tumor cells, suggesting that the drug may have beneficial immunomodulating effects in addition to its known direct toxicity.

MATERIALS AND METHODS

Animals, Tumors, Chemicals, and Reagents. C57BL/6 and DBA/2 mice, 6 to 10 weeks old, were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA. P815 mastocytoma cells were maintained in an ascitic form in adult DBA/2 mice and used as target cells in in vivo cytostasis assays.

Bisantrene (CL 216,942; NSC 337766) was obtained from Dr. K. C. Murdock, Lederle Laboratories. Its solubility is 50 µg/ml in water at pH 7.4 at 23°C. For administration to animals, drug was suspended in a sterile 0.3% Klucel solution (Hercules, Inc., Wilmington, DE) in Dulbecco's phosphate-buffered saline at the desired concentrations, and 0.5 ml was injected i.p. into each mouse. Bisantrene preparations contained no endotoxin as indicated by the Limulus amebocyte lysate test.

HBSS, RPMI 1640, fetal calf serum, l-glutamine, penicillin, streptomycin, Dulbecco's phosphate-buffered saline, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid were obtained from Grand Island Biological Company, Grand Island, NY. RAMTC antibody and Low-Tox-M rabbit complement were obtained from Cedarslane Laboratories, Westbury, NY. Gentamicin was obtained from Schering Corp., Kenilworth, NJ; heparin sodium was obtained from The Upjohn Co., Kalamazoo, MI; thiglycolate medium was obtained from Difco Laboratories, Detroit, MI. TLCK, aprotinin, catalase, carrageenan, and trichloroacetic acid were obtained from Sigma Chemical Co., St. Louis, MO. [3H]dThd (specific activity, 20 Ci/mmol) was obtained from New England Nuclear, Boston, MA.

Preparation of Murine Macrophages. C57BL/6 mice were given i.p. injections of 1 ml of thiglycolate medium, and the PE cells were harvested 4 to 5 days later by washing their peritoneal cavities with HBSS containing heparin, (10 units/ml). PE cells were washed 3 times with HBSS and suspended in RPMI 1640 containing 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml), 2 mM l-glutamine, and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (referred to as complete medium). After determination by viable and differential cell counts, appropriate numbers of macrophages were dispensed into the flat-bottomed wells of a 96-well culture plate.
plate (Costar, Cambridge, MA). After incubation at 37° for 2 hr, nonadherent PE cells were removed by repeated and vigorous washings with HBSS. When these recovered nonadherent cells were counted, approximately 40 to 55% of the initial added cells remained in culture wells, and the majority of these cells (≥95%) morphologically resembled macrophages. These cells were also functionally active in ingesting latex particles. Unless otherwise noted, experimental macrophages were similarly prepared from PE cells harvested from mice given both bisantrene and thioglycolate. The compound at the dose of 100 mg/kg or less caused no apparent toxicity since ≥99% harvested PE cells were viable and about 50% of these cells were capable of adhering to plastic.

In Vitro and in Vivo Treatments with Carrageenan. Carrageenan was initially dissolved in Dulbecco’s phosphate-buffered saline at 1 mg/ml and diluted in complete medium to an appropriate concentration prior to use. After being incubated with carrageenan (125 to 500 μg/ml) at 37° for 2 hr, adherent PE cells were washed with HBSS 3 times before further testing. This procedure caused no alternation of cell viability as judged by trypan blue dye exclusion. Although our observation was in agreement with that of Catanzaro et al. (5), who showed no toxicity to murine macrophages by carrageenan at a concentration of 2 mg/ml, Allison et al. (3) indicated a slow damage of macrophages by carrageenan (200 μg/ml).

For in vivo studies, mice were given i.p. injections of 0.75 mg of carrageenan on Days −4 and −1, and PE cells were harvested on Day 0. Macrophages for cytostasis assays were prepared from these mice by the method described above.

Treatment of Effector Cells with RAMTC Antibody and Complement. PE cells were incubated with RAMTC antibody (1:20 dilution) at 4° for 60 min following plastic adherence. These cells were then exposed to rabbit Low-Tox-M complement (1:10 dilution) and incubated at 37° for an additional 60 min. These cells were washed 3 times with HBSS prior to use. This procedure was previously shown to deplete T-lymphocytes, since treated cells no longer responded to concanavalin A, whereas their lipopolysaccharide response remained intact.

Assay for Macrophage-mediated Tumor Cytostasis. P815 tumor cells obtained from DBA/2 mice were washed twice with HBSS and suspended in complete medium. P815 cells (5 × 10⁶) were added to culture wells containing macrophages, and the E:T ratios were established on the basis of initial numbers of macrophages added to each well. Unless otherwise noted, culture plates were incubated at 37° in a 7% CO₂ and water-saturated atmosphere for 2 days. Cells in each well were pulsed with 0.5 μCi [³H]dThd for the final 4 hr and harvested with a cell harvester. Cellular DNA was precipitated on glass fiber filters with 10% trichloroacetic acid, and the amount of [³H]dThd incorporated was determined in a liquid scintillation counter. The mean cpm were obtained from triplicate cultures, and results were presented as percentage cytostasis which was calculated by the formula

\[
\text{% of cytostasis} = \left( \frac{A - B}{A} \right) \times 100
\]

where \(A\) is cpm of cultures containing normal control macrophages, and \(B\) is cpm of cultures containing experimental macrophages. The significance of the difference between the effects of control and experimental macrophages was determined by Student's t test. Results reported here are representative of 1 to 4 experiments.

RESULTS

Induction of Tumor-cytostatic Macrophages In Vivo. Table 1 presents the results of a typical experiment to illustrate the effect of bisantrene on activating macrophages. As compared to cultures of P815 tumor cells alone that remained in log-phase growth throughout incubation, macrophages from untreated mice or mice given i.p. injections of 1 ml thioglycolate or 0.3% Klucel did not significantly alter the growth of tumor cells. However, macrophages from mice treated with bisantrene (100 mg/kg) almost completely inhibited tumor cell proliferation. Since control macrophages used in this report were prepared from mice given injections of thioglycolate, bisantrene-treated mice also received thioglycolate. Thioglycolate itself had no effect on macrophage activation as determined by cytostatic effects on tumor cells in vitro (Table 1). A dose-response study was subsequently carried out by giving mice i.p. injections of doses of bisantrene ranging from 25 to 200 mg/kg. Macrophages were prepared from these mice 4 days later, and their activity was tested in vitro at an E:T ratio of 20:1. Macrophages from mice receiving bisantrene (25 mg/kg) showed a significant antiproliferative effect (percentage of cytostasis, 37.6; \(p < 0.05\)), but the most profound activity (~80% cytostasis) was clearly seen with cells from mice treated with 50 mg/kg or more (Chart 1). The optimal macrophage-stimulating dose appears to be somewhat lower than the optimal antitumor dose determined to be about 150 mg/kg against P388 leukemia and B16 melanoma in mice.

The time course of activation of macrophages by bisantrene was investigated by testing macrophages harvested from mice at various time intervals (2 days to 4 weeks) after receiving 100- to 200-mg/kg doses of drug. The inhibitory effect of these macrophages on tumor cell growth was tested at an E:T ratio of 5:1. Results presented in Chart 2 demonstrate that extremely effective macrophages appeared in mice as early as 2 days after treatment and persisted for at least 4 weeks (\(p < 0.001\)).

### Table 1

<table>
<thead>
<tr>
<th>Cultivation of tumor cells with</th>
<th>cpm</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>30093.6 ± 2880.4</td>
<td>NS²</td>
</tr>
<tr>
<td>Resident macrophages</td>
<td>32564.4 ± 5569.1</td>
<td>NS²</td>
</tr>
<tr>
<td>Thioglycolate-treated macrophages</td>
<td>33357.6 ± 1385.4</td>
<td>NS</td>
</tr>
<tr>
<td>Klucel-treated macrophages</td>
<td>23354.1 ± 5352.3</td>
<td>NS</td>
</tr>
<tr>
<td>Bisantrene-treated macrophages</td>
<td>276.0 ± 93.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Bisantrene³ and thioglycolate-treated macrophages</td>
<td>268.7 ± 36.4</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

- P815 cells (5 × 10⁶) were cocultured with or without macrophages from various treated mice. Proliferation of tumor cells was determined 48 hr later by the incorporation of [³H]dThd. E:T, 10:1.
- Mean ± S.D.
- NS, not statistically significant.
- Mice were treated with bisantrene (100 mg/kg) 4 days earlier.

![Chart 1. Dose-response effect of bisantrene on the activation of macrophages.](chart)

Mice were treated with various doses of bisantrene and their macrophages were prepared 4 days later for testing for tumor cytostasis at an E:T ratio of 20:1. * \(p < 0.05\); ** \(p < 0.01\); *** \(p < 0.001\).
Macrophage Activation by Bisantrene

Chart 2. Persistence of activated macrophages following a single dose of bisantrene. Macrophages were prepared from mice at various time intervals after treatment with bisantrene (100 mg/kg). These macrophages were tested for tumor cytostasis at an E:T ratio of 5:1. ***, p < 0.001.

Chart 3. Activation of macrophages with bisantrene. Mice were treated i.p. with bisantrene (50 or 100 mg/kg). Macrophages were prepared 4 days later and tested for tumor cytostasis at various E:T ratios. **, p < 0.01; ***, p < 0.001.

Optimization of Tumor Cytostasis by Effector Macrophages. Macrophages were prepared from drug-treated mice and tested for inhibition of tumor cell growth at various E:T ratios (20:1 to 0.5:1). Macrophages from mice receiving 50-mg/kg doses of drug was found to be significant at the higher E:T ratios (20:1 to 2:1).

The optimal period of incubation was studied by coculturing effector and target cells at an E:T ratio of 5:1 for various lengths of time. Although a significant effect of experimental macrophages became detectable as early as 24 hr after initiation of cultures (percentage of cytostasis, 26.5; p < 0.01), incubation for 48 and 72 hr markedly increased the percentage of cytostasis to 89.4 and 95.5, respectively (p < 0.001) (Chart 4). The 48-hr incubation produced near optimal results and for convenience was used throughout this study.

Effect of Culture Supernatants of Activated Macrophages. Macrophages prepared from mice 5 days after treatment with bisantrene (100 mg/kg) were cultured in vitro for 2 days. Supernatants were harvested, filtered through an 0.45-μm Millipore filter, and added to P815 tumor cell cultures at various dilutions. The proliferation of tumor cells was determined 2 days later by [3H]dThd incorporation. Results in Chart 5 clearly demonstrate that these supernatants inhibited the growth of P815 cells in a dose-dependent fashion, with maximal cytostasis being achieved by a 5% concentration of supernatant. The presence of cytostatic factor(s) produced by activated macrophages was indicated.

Characterization of Effector Cells. Results obtained thus far indicate that the effector cells being investigated resembled macrophages morphologically. In an attempt to better define the cell type involved in the cytostatic reaction, PE cells were treated with carrageenan or RAMTC antibody following plastic adherence purification. As indicated in Table 2, 250- and 500-μg/ml doses of carrageenan completely abolished the cytostatic effect of the PE effector cells. A partial but significant decrease of inhibition of tumor cell growth also was seen with carrageenan (125 μg/ml). Inactivation of effector cells with carrageenan was also evident following in vivo treatment (Table 3). On the other hand, incubation with RAMTC antibody and complement failed to alter the effect (Table 2). Taken together, these findings strongly suggested that bisantrene acted upon macrophages rather than on T-lymphocytes.
Identification of Cytostatic Factor(s) Produced by Activated Macrophages. Various compounds were added to cultures in an attempt to identify possible mediators produced by activated macrophages. As presented in Table 4, addition of 2 protease inhibitors, TLCK and aprotinin, to cultures did not influence macrophage activity, suggesting that neutral proteases were not involved. Likewise, failure of catalase to alter the cytostatic effect indicated that hydrogen peroxide did not participate in this reaction. In order to be certain of the activity of the reagents used, we tested them in another system. Macrophages from mice treated with Bacillus Calmette-Guérin 10 days earlier showed a significant activity against P815 targets [percentage of cytostasis, 57.5 ± 2.8 (S.E.)]. However, the effect was almost completely abrogated by the presence of TLCK (5 x 10^{-5} M), aprotinin (0.5 trypsin inhibitor unit/mL), and catalase (1000 units/mL) as indicated by their percentages of cytostasis, 0.5 ± 0.4, 0, and 5.0 ± 2.3, respectively.

In order to determine whether or not reduced [3H]dThd incorporation was resulted from a competition with cold thymidine possibly released by macrophages, a cytostasis assay was set up as usual. However, after a 48-hr incubation, target cells were transferred from microwells to culture tubes and were washed thoroughly prior to exposure to [3H]dThd for the final 4-hr incubation. Again, a profound activity was clearly observed with bisantrene-activated macrophages (percentage of cytostasis, 98.7 ± 0.4 and 91.1 ± 0.8 at E:T ratios of 10:1 and 5:1, respectively).

**DISCUSSION**

Bisantrene is a novel antineoplastic agent shown to be effective against several experimental (6) and human (26, 28) cancers. Although its precise mechanism of action is incompletely defined, preliminary studies have indicated that the drug intercalates with DNA (4, 27). The present findings seem to suggest further that potentiation of tumor-cytostatic macrophages in vivo may also contribute to the antitumor activity of bisantrene, but this possibility awaits confirmation.

In this study, macrophages from mice treated with bisantrene were found to be extremely active in preventing the proliferation of P815 mastocytoma cells in vitro. Significantly effective in vivo doses ranged from 25 to 200 mg/kg. Effector macrophages were detected in animals 2 days after treatment and persisted for at least 4 weeks, indicating a long-lasting effect. The activity of these macrophages was so profound that significant effects were unexpectedly detectable at E:T ratios as low as 0.5:1. Incubation of activated PE cells with target cells for 48 hr seemed necessary to achieve the maximum effect and also indicated that T-cells and natural killer cells were probably not the effector cells.

The defense mechanism of animals consists of several types of immunocompetent cells. Macrophages apparently were the primary targets of bisantrene since the effect was significantly abolished by both in vitro and in vivo treatment with carrageenan, which is known to abrogate macrophage activity (5). In contrast,
as a result of insensitivity to treatment with RAMTC antibody and complement, T-lymphocytes were unlikely to be responsible for the observed results. Finally, P815 cells used as targets in this study have been shown by others to be resistant to natural killer cell activity (21), thus ruling out the possibility that natural killer cells accounted for the findings presented here.

Macrophages have been well documented to affect the growth of neoplastic cells after being activated by a variety of substances including Bacillus Calmette-Guérin (11), Corynebacterium parvum (18), endotoxins (2), muramyl dipeptide (10), polyinosinic-polycytidylic acid (2), pyran copolymer (12), lymphokines (22), Adriamycin (14, 25), cyclophosphamide (25), and mitomycin C (17). It seems that the list of macrophage stimulants can be extended to include bisantrene. Although the mechanism by which bisantrene-activated macrophages destroy tumor targets needs further clarification, the release of toxic mediators such as hydrogen peroxide (16) and/or neutral proteases (1) was not responsible since tumor cytostasis was not altered by the addition of catalase, TLCK, and aprotinin to cultures. Antiproliferative effects seen with culture supernatants suggested that a cytostatic factor or factors might be produced by these activated macrophages. Experiments in which target cells were washed prior to being pulsed with [3H]dThd indicated that free thymidine possibly produced by macrophages (19) played no rule in our system. Whether or not this factor(s) relates to arginase (7), C3a complement (9), cytoplasmic lysozymes (11), cytotoxic (20), tumor necrosis factor (15), or toxic factors described by others (8, 23) remains to be answered. Alternatively, bisantrene might be temporarily bound to and retained by macrophages and subsequently released into cultures causing a direct inhibition of tumor cell growth. This explanation seems unlikely, however, because the amount of bisantrene as measured by the release of 14C-labeled drug in culture supernatants was insufficient to account for the present results (data not shown).

Clinical results achieved with immunotherapy of neoplastic disorders have been disappointing, and future attempts may perhaps lie in determining its role in combined modality approaches. As a result of its unique properties of both direct tumor toxicity and macrophage activation, bisantrene may prove clinically manipulable as a single immunochemotherapeutic agent for the treatment of cancer.

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REFERENCES

Activation of Tumor-cytostatic Macrophages with the Antitumor Agent 9,10-Anthracenedicarboxaldehyde Bis[(4,5-dihydro-1 H -imidazole-2-yl)hydrazone] Dihydrochloride (Bisantrene)


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