Inhibition of Cellular Division of a Murine Macrophage Tumor by Macrophage-activating Agents

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ABSTRACT

Although the murine reticulum cell sarcoma M5076 is highly malignant in vivo, in vitro it displays many of the functional characteristics of an activated macrophage, such as phagocytosis and tumor cytotoxicity. This study was designed to determine what effect macrophage-activating agents would have on the function and growth of M5076 cells.

Exposure of M5076 tumor cells to substances that activate normal macrophages to the tumoricidal state rapidly and irreversibly induced cessation of cellular division. The treated tumor cells, however, retained the same characteristics as those of untreated M5076 cells in vitro with respect to viability and the activated macrophage functions of phagocytosis and tumor cytotoxicity. Even after a short exposure to lipopolysaccharide, the ability of M5076 cells, injected i.v. into syngeneic mice, to form tumor nodules was greatly reduced.

These results indicate that a highly malignant tumor of macrophage origin, M5076, can be induced to cease cellular division while retaining the functional attributes of an activated macrophage. We speculate that the exposure of M5076 to macrophage-activating agents results in the induction of terminal differentiation of this tumor.

INTRODUCTION

Studies of the mechanisms of cell growth and differentiation in both normal and tumor cells have been facilitated greatly by the establishment of cell lines that progress to terminal differentiation in the presence of the appropriate inducing agents (16, 21, 23, 28, 29). Cells of the erythroid and myeloid series, which express distinct biochemical and morphological markers at various stages of differentiation, have been particularly useful in these investigations (2, 9, 13, 19, 20). A correlation between the malignant behavior of these cell lines and a poor degree of differentiation has been established; e.g., the least differentiated cells are the most malignant (6, 7). Furthermore, agents that are capable of inducing terminal differentiation in vivo may be therapeutically effective in preventing tumor growth in experimental animals inoculated with these tumors (8, 12, 26).

Macrophages can be activated in vitro and in vivo with regard to phagocytosis, enzyme synthesis and secretion, membrane activity, metabolism, antibacterial activity, and antitumor capacity (5). Such activated macrophages may represent a final stage of differentiation in cells of the mononuclear phagocyte series. Many of the agents that activate macrophages, such as LPS, TPA, and double-stranded polyribonucleotides (polyninosinic-polycytidylic acid) (1, 5, 10), have also been shown to stop growth and to induce differentiation in myeloid leukemic cells (8, 9, 12, 19, 26, 27, 30). We have described recently a murine reticulum cell sarcoma, M5076, which is highly malignant in vivo but which exhibits the functional and biochemical characteristics of an activated macrophage in vitro (4, 25).

The present study was undertaken to determine how cells of this tumor line would respond in vitro and in vivo to substances that are capable of activating normal macrophages and acting as differentiation stimulants in other systems. Because the M5076 tumor displays most of the phenotypes of the activated macrophage, we were interested in determining whether exposure to the exogenous stimuli that usually produce activation would lead to a more activated state. We report here that, after TPA and LPS treatment, M5076 cells lose the ability to divide in vitro as assessed by [3H]thymidine incorporation and cell counts and have a reduced ability to form tumors in vivo.

MATERIALS AND METHODS

Animals. Specific-pathogen-free female C57BL/6N X C3H F, (hereafter called B6C3F1) mice, 6 weeks old, were obtained from the Animal Production Area, Frederick Cancer Research Facility. The mice were age matched within each experiment.

Cells. M5076 murine reticulum sarcoma cells were grown as described previously (21) in RPMI Medium 1640 and 10% heat-inactivated equine serum. The cells were free of Mycoplasma, reovirus type 3, pneumonia virus, K virus, Thielier’s virus, Sendai virus, minute virus, mouse adenosivirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (M. A. Bioproducts, Walkersville, Md.).

TPA and 12-O-tetradecanoylphorbol-13-acetate (P-L Biochemicals, Inc., Milwaukee, Wis.) were solubilized in acetone and added to the cell suspensions as detailed in Results. Control cultures received equivalent amounts of acetone. LPS from Escherichia coli 055:BS (Difco Laboratories, Detroit, Mich.) was dissolved in RPMI Medium 1640 (Grand Island Biological Company, Grand Island, N. Y.) containing 10% equine serum and was added to the various assays as noted in Results.

Phagocytic Activity. The incorporation of opsonized, 51Cr-labeled sheep RBC by 106 M5076 tumor cells was determined for control cells and for cells that had been pretreated by incubation for 24 or 96 hr in media containing 5 μg LPS per ml using methods described elsewhere (21).

DNA Synthesis. M5067 cells were plated in 96-well plates at 104 cells/well in 0.2 ml of medium. After 24, 48, or 72 hr of incubation, the cells were labeled for 2 hr with 1 μCi of [3H]thymidine (specific activity, 55 Ci/mmol). The cells were harvested and lysed in water to remove

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: LPS, lipopolysaccharide; TPA, 12-O-tetradecanoylphorbol-13-acetate; RPMI, Roswell Park Memorial Institute; FITC, fluorescein-isothiocyanate-labeled.

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soluble pools of [3H]thymidine using a MASH II harvester. Incubation conditions are described in "Results."  

Tumoricidal Activity In Vitro. The tumoricidal activity of M5076 effector cells against B16 melanoma target cells (3) was assayed as described previously (25).

Experimental Metastatic Capacity. The malignant capacity of the M5076 cells was measured by the experimental metastasis assay (4). Adult mice, syngeneic to the M5076 tumor, were given i.v. injections of single-cell suspensions (> 95% cell viability as assessed by trypan blue exclusion) of equal numbers of cells pretreated with LPS (see "Results") and control tumor cells. Both cell populations consisted of single cells in suspension with no cellular aggregation. Three weeks after tumor inoculation, the mice were killed and necropsied. The liver was removed and fixed in Bouin's fixative, and the superficial hepatic tumor nodules were counted with the aid of a dissecting microscope.

Electron Microscopy. Cells in suspension were fixed in 2.5% glutaraldehyde in 0.5 M cacodylate buffer (pH 7.3) for 1 hr. The cells were centrifuged and washed twice with buffer, and the pellet was resuspended in 1 ml of buffer. Cells were allowed to adhere to polylysine-coated glass coverslips for 1 hr at 4°. The coverslips were washed, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 1 hr, and incubated in control medium (without LPS) for the indicated time before [3H]thymidine pulsing. Values given are means of quadruplicate cultures (S.D. < 10%).

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0 (15,734 ± 4,173)*</td>
<td>0 (23,603 ± 4,746)</td>
<td>0 (28,329 ± 3,179)</td>
</tr>
<tr>
<td>LPS</td>
<td>25 μg/ml</td>
<td>97 (509 ± 183)</td>
<td>97 (787 ± 93)</td>
</tr>
<tr>
<td>5 μg/ml</td>
<td>97 (502 ± 259)</td>
<td>98 (427 ± 174)</td>
<td>98 (604 ± 176)</td>
</tr>
<tr>
<td>0.005 μg/ml</td>
<td>91 (1,456 ± 349)</td>
<td>83 (4,084 ± 796)</td>
<td>74 (7,408 ± 1,816)</td>
</tr>
<tr>
<td>Control</td>
<td>0 (7,940 ± 1,276)</td>
<td>0 (18,528 ± 3,121)</td>
<td>0 (23,969 ± 2,178)</td>
</tr>
<tr>
<td>TPA</td>
<td>50 ng/ml</td>
<td>89 (888 ± 115)</td>
<td>92 (1,416 ± 112)</td>
</tr>
<tr>
<td>12.5 ng/ml</td>
<td>89 (436 ± 70)</td>
<td>90 (1,807 ± 306)</td>
<td>90 (2,391 ± 57)</td>
</tr>
<tr>
<td>6 ng/ml</td>
<td>92 (655 ± 187)</td>
<td>83 (3,221 ± 281)</td>
<td>87 (3,201 ± 816)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, mean ± S.D. of cpm obtained from triplicate cultures. Additional triplicate cultures were terminated at 48 and 72 hr; viability was assessed by trypan blue exclusion (range, 81 to 98%) and was not correlated with inhibition of DNA synthesis.

RESULTS

LPS- and TPA-induced Inhibition of [3H]Thymidine Incorporation. Incubation of the murine M5076 cells with varying concentrations of LPS or TPA produced marked arrest in cell proliferation as demonstrated by the inhibition of [3H]thymidine incorporation (Table 1) and substantiated by direct cell counts (Table 2). The effect of LPS treatment appeared to be mediated via the lipid A moiety because polymyxin B, which binds irreversibly to lipid A (15), totally abrogated the LPS-induced inhibition of DNA synthesis and returned the [3H]thymidine incorporation to 100% of the control level. Incubation of M5076 cells in LPS (5 μg/ml) for as little as 10 min produced almost as great an inhibition of [3H]thymidine incorporation as did continuous culture of M5076 cells for 24 hr in the same concentration of LPS (Chart 1). Inhibition of DNA synthesis was irreversible because M5076 cells incubated in LPS (5 μg/ml) for 24 hr, washed repeatedly, and then incubated for up to 7 days in fresh medium failed to incorporate [3H]thymidine (Table 2). Moreover, no increase in cell number could be detected by direct cell counts although viability remained high (Table 2). Because incorporation of [3H]thymidine correlated with cell number, it is unlikely that the reduced incorporation of radiolabeled thymidine was a consequence of increased thymidine production by the M5076 cells (24) with subsequent inhibition of [3H]thymidine uptake.

Binding and Internalization of LPS. LPS binds rapidly and avidly to cell membranes. Thus, it is possible that the decreased DNA synthesis of M5076 cells following exposure to LPS was the result of LPS that remained membrane bound throughout the extensive washing or that rebound to the cells during the incubation period. To study this question, we used fluorescent-labeled LPS (List Biological Laboratories, Inc., Campbell, Calif.) to monitor LPS binding. M5076 cells were incubated with FITC-LPS (1 μg/ml) for 1 hr and then were washed thoroughly with medium containing polymyxin B (2 μg/ml) to prevent rebinding of the LPS. Aliquots of the cell suspension were then trypsin-
ized (0.25% trypsin-0.02% EDTA) for 5 min at 37°C and washed twice with medium containing polymyxin B. These cells were cultured, as were control cells, in 96-well plates (10^4 cells/well in 0.2 ml) and pulse-labeled with [3H]thymidine. As shown in Table 3, trypsinization did not reverse the inhibition of DNA synthesis and had only a slight effect on the DNA synthesis of the trypsinized control cultures.

The question of whether failure of trypsinization to reverse LPS-induced inhibition of DNA synthesis was due to the rapid uptake of LPS was studied using the fluorescent-labeled LPS. As demonstrated in Fig. 1, only a small amount of fluorescent LPS remains bound to the membrane of M5076 cells following a 1-hr incubation with FITC-LPS and subsequent thorough washing, whereas large amounts of LPS had been rapidly internalized and were apparent within cytoplasmic vacuoles in this time period (Fig. 1).

Chart 1. Rapid and irreversible nature of LPS-mediated inhibition of DNA synthesis. M5076 cells were exposed to LPS (5 μg/ml) for 10 min or continuously and then incubated for the appropriate time after repeated washing of the cells given the 10-min LPS pulse. Bars, mean values from quadruplicate cultures (S.D. < 10%).

Table 3. Incorporation of [3H]thymidine by M5076 cells incubated with LPS for 1 hr.

<table>
<thead>
<tr>
<th>Treatment of M5076 cells</th>
<th>[3H]Thymidine incorporation (cpm)</th>
<th>% of inhibition of [3H]thymidine incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control M5076 cells</td>
<td>25,003 ± 2.71</td>
<td></td>
</tr>
<tr>
<td>Control M5076 cells with trypaning</td>
<td>16,640 ± 853</td>
<td></td>
</tr>
<tr>
<td>LPS-treated M5076 cells</td>
<td>797 ± 209</td>
<td>97</td>
</tr>
<tr>
<td>LPS-treated M5076 cells with trypaning</td>
<td>556 ± 115</td>
<td>97</td>
</tr>
<tr>
<td>FITC-LPS-treated M5076 cells</td>
<td>595 ± 99</td>
<td>98</td>
</tr>
<tr>
<td>FITC-LPS-treated M5076 cells with trypaning</td>
<td>190 ± 88</td>
<td>99</td>
</tr>
</tbody>
</table>

<sup>a</sup> M5076 cells were incubated with LPS (1 μg/ml) or FITC-LPS for 1 hr, washed, trypsinized for 5 min, and rewashed. Washes were with medium containing polymyxin B which irreversibly binds to the active site of LPS and prevents rebinding. The cells, 10,000 in 0.2 ml, were cultured in 96-well plates for 24 hr, pulsed with [3H]thymidine, and harvested using a water lysis to remove soluble, unincorporated label.

<sup>b</sup> Percentage of inhibition of [3H]thymidine incorporation as compared to the appropriate control, with or without trypsinization.

<sup>Mean ± S.D.</sup>

**Scanning Electron Microscopy.** Normal M5076 tumor cells (Fig. 2a) are very similar to activated peritoneal exudate cells obtained by i.p. injection of *Corynebacterium parvum* (Fig. 2c). Both cell types exhibit marked membrane ruffling and blebbing with few microvilli. Incubation of M5076 cells in LPS (Fig. 2b) led to extensive microvilli formation and a loss of membrane ruffles; this is similar to the appearance of unactivated resident peritoneal exudate cells obtained after the i.p. injection of thioglycollate (Fig. 2d).

**Functional Capacity of LPS-treated M5076 Cells.** The functional capacity of M5076 cells, as measured by phagocytic activity and the ability to lyse 5-[3H]iodo-2'-deoxyuridine-labeled B16 melanoma cells, was unaltered after incubation in levels of LPS that almost completely inhibited DNA replication. Antibody-coated sheep RBC were incubated for 60 min with control or M5076 cells pretreated with LPS (5 μg/ml); after incubation, nonphagocytosed erythrocytes were lysed with water. No statistical differences in phagocytic abilities were found between control and LPS-treated M5076 cells. Similarly, the preincubation of M5076 cells in LPS or the addition of...
macrophage-activating agents in vitro and speculate that this effect of such a brief exposure is fully apparent only after 24 hr additional incubation, suggesting that LPS may act to block DNA synthesis at a specific point in the cell cycle. We are currently investigating this aspect of these findings. When such LPS-pretreated cells were injected i.v. into recipient mice, they exhibited a marked reduction in malignant capacity as demonstrated by a reduction in the number of hepatic foci produced (Table 5).

From these data, we conclude that a highly malignant cell line can be induced to cease cellular division by exposure to macrophage-activating agents in vitro and speculate that this represents the induction of terminal differentiation. This conclusion is based on the evidence of irreversible inhibition of cellular proliferation as assessed by DNA synthesis and cell counts and on alterations in surface morphology accompanied by the maintenance of viability and functional capacity normally associated with activated macrophages.

Unlike the myeloid leukemia cell lines commonly used in studies on differentiation (9, 19, 30), the M5076 cells are highly malignant in vivo and express a number of functions normally associated with differentiated, activated (tumoricidal) macrophages in vitro. Indeed, one of the problems in determining whether the macrophage-activating agents induce terminal differentiation in M5076 cells is that all the functional markers characteristic of activated macrophages are already present in the dividing M5076 tumor cells (25) and are not altered after differentiation is induced. Recently, Sachs (22) has proposed that the expression of certain genes in malignant cells may be altered from inducible to constitutive and that this may, in some way, interfere with the ability of the cells to proceed to terminal differentiation. An analogy may be drawn from this that, in M5076 tumor cells where the expression of the activated phenotype has become constitutive as compared to inducible, the progress to terminal differentiation has been blocked and may then be induced by macrophage-activating agents.

Ralph and Nakoinz (17) have also shown that the growth of macrophage cell lines adapted to culture was inhibited by various macrophage-activating agents. Furthermore, in agreement with the findings presented here, such growth inhibition did not affect the differentiated functions of the tumor lines (17). However, in contrast to our results, these workers found that such growth inhibition was rapidly reversible following removal of the agent. Because these investigators did not achieve irreversible cessation of cell growth, they believed that their observations did not correlate with the induction of terminal differentiation in the macrophage cell lines studied. This,
however, does not appear to be the case with the M5076 tumor system studied here and suggests that different mechanisms may be operative in different tumor lines.

We have been interested in the concept of driving malignant cells toward terminal differentiation as a therapeutic modality (8, 12, 26). In preliminary experiments with M5076 tumor-bearing animals, we have been able to achieve a reduction in metastatic burden by the i.v. injection of LPS. In one such experiment, syngeneic mice given i.v. injections of 25,000 M5076 cells had a median of >400 (range, 1 to >400) hepatic tumor foci 28 days after tumor injection. A single i.v. injection of 1 μg LPS 1, 3, or 7 days following the injection of M5076 tumor cells reduced the median number of hepatic tumor foci to: 0 (range, 0 to 28); 0 (range, 0 to 1); and 2.5 (range, 0 to 40), respectively. We ascribe this reduction in tumor foci to the induction of differentiation but, because the putative differentiation agents studied also activate macrophages, additional studies are required to rule out the possibility that such agents act by stimulating the host mononuclear phagocytic system. The possibility that the tumors that develop during this therapy may lack the appropriate receptors for the inducing agents is also being investigated. Such variants that cannot be induced to differentiate have also been reported from the WEHI-3B myelomonocytic leukemia tumor line (11) and a myeloid leukemia tumor line from an SL mouse (14). The existence of such nondifferentiating variants and the parent line of the M5076 tumor will provide a useful model system for studying the interrelationships of growth control, cellular differentiation, and malignancy in a solid tumor system.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 2. Scanning electron micrographs of M5076 tumor cells and murine peritoneal macrophages. a, tissue-culture-propagated M5076 cell maintained in RPMI Medium 1640 as described in text. The cell shows extensive membrane ruffling comparable to that of an activated peritoneal macrophage (b) obtained by the i.p. injection of C. parvum. Following incubation in medium containing LPS (5 μg/ml), the M5076 cells (c) show an increase in size and a loss of membrane ruffling. There are extensive microvilli similar to those found in the unactivated peritoneal macrophage (d) obtained by i.p. injection of thioglycollate.
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