ABSTRACT

The effects of the differentiation agent, N,N-dimethylformamide (DMF), on malignant AKR-MCA cells were studied. The properties of DMF-treated AKR-MCA cells were compared to those of the normal parental AKR-2B mouse embryo fibroblasts. AKR-MCA cells grown in 1% DMF were found to be more similar to their normal counterparts than to untreated AKR-MCA cells by several criteria. These criteria included the loss of the transformed morphology, a 2-fold reduction of doubling time, a 10-fold reduction of saturation density, and the complete loss of the ability to grow with anchorage independence.

The expression of high-molecular-weight membrane antigens (M, 110,000 to 450,000), which was found to be greatly reduced in AKR-MCA cells in comparison to normal AKR-2B cells, was restored by treatment of AKR-MCA cells with DMF. The expression of a low-molecular-weight AKR-MCA cell-associated membrane antigen, on the other hand, was found to be suppressed.

Studies on the mitogenic response of these cells indicated that AKR-MCA and AKR-2B cells may be regulated by different types of growth control. Growth-arrested AKR-MCA cells did not respond to epidermal growth factor, while their ability to respond to nutrient replenishment was lost. The results of this study indicated that DMF treatment induced the normalization of malignant AKR-MCA cells with regard to membrane antigen composition and growth control properties.

INTRODUCTION

Planar polar reagents such as sodium butyrate, dimethyl sulfoxide, and DMF3 have been used to induce terminal differentiation of leukemic cells (11, 19, 26). More recently, the effects of these compounds on the growth of solid tumors in vitro have been studied. Kim et al. (17, 21, 30) have described the induction of several molecular alterations in colorectal tumor cells which may be indicative of a more differentiated state of cells treated with planar polar reagents. Dexter et al. (6, 7) have described alterations in morphology, growth characteristics, and immunological markers of colorectal tumor cells which also suggest the induction of a more differentiated phenotype by DMF. The inhibitory effects of DMF and monomethylformamide on the growth of tumors in athymic nude mice have been reported, and

methylformamide, a human metabolite of DMF, is currently undergoing clinical trial (8). The appearance of differentiation markers in solid tumor systems in vitro suggests that treatment of malignant cells with planar polar reagents induces a more "normalized" phenotype in the target cells but, in contrast to leukemic cells, terminal differentiation does not occur.

One difficulty in interpreting the degree of "normalization" induced by planar polar agents in malignant cells which retain proliferative ability is the lack of untransformed normal counterpart cells in vitro. Related to this is the difficulty in assessing whether a malignant target cell has been reprogrammed by epigenetic stimuli to express more normal phenotypes, or whether the expression of differentiation markers in treated cells is associated with the reduction of cell proliferation resulting from sublethal damage.

We have attacked these problems in a system in which the normal counterpart to the malignant cell was available in tissue culture. The system we selected for evaluation consisted of mouse embryo AKR-2B fibroblasts and their methylcholanthrene-transformed counterparts, designated AKR-MCA cells. These cells have been thoroughly characterized by Getz et al. (12), Moses et al. (22, 23), and Robinson et al. (25). AKR-2B cells have a normal fibroblastic morphology, do not grow with anchorage independence, and are not tumorigenic in athymic nude mice. AKR-MCA cells have a transformed morphology, grow in semisolid medium, and form tumors in athymic nude mice. The 2 types of cells are also under different types of growth control. Growth-arrested AKR-MCA cells, like other chemically transformed cells (23), do not respond to exogenously added growth factors such as EGF. On the other hand, growth-arrested normal AKR-2B cells will respond to exogenous factors. It is likely that the lack of response by AKR-MCA cells to exogenous factors is due to the production of autostimulatory factors by these cells (31).

This report describes the effects of DMF on AKR-MCA cells. Treatment of AKR-MCA cells with 1% DMF resulted in the normalization of the transformed cells with respect to morphology, anchorage-independent growth, and membrane antigen composition. We also report that treatment of malignant cells with DMF restored the normal mitogenic response to exogenous EGF exhibited by untransformed cells.

MATERIALS AND METHODS

Cell Culture. AKR-2B and AKR-MCA cells were obtained from Dr. H. L. Moses of the Mayo Clinic, Rochester, MN. The cells were maintained in McCoy's Medium 5A supplemented with 10% FBS and antibiotics (streptomycin-penicillin). Growth curves were performed as described previously (2). Briefly, cells were inoculated into 25-sq cm flasks (Coming) on Day 0 in 5 ml of the growth medium described above. On Day 1, the growth medium was changed to 5 ml of growth medium containing 1% DMF (Sigma) and 10% FBS or plain growth medium in control flasks.
Transferred proteins were similarly treated with preimmune sera or with material was used to determine DNA synthesis as described by Benz et al. (1). Cells were grown to confluence in 9-sq cm, 6-well plates (Falcon) in standard growth medium containing 10% FBS as described above. At confluence, standard growth medium was replaced with medium containing 0.5% FBS. After \(^{3}H\)thymidine incorporation was stabilized at a minimum (~24 hr) during growth arrest, EGF (16 ng/ml) was added to the cells. Controls were treated with fresh medium lacking EGF. After 20- to 22-hr incubation, \(^{3}H\)thymidine (2.5 mCi/well; 0.7 \(\mu\)Ci/ml) was added, and the cells were incubated for an additional hr at 37°. Cells were washed and \(^{3}H\)thymidine uptake, and incorporation into acid-insoluble counts was determined as described by Benz et al. (1). Samples treated identically, except for the lack of thymidine addition, were used for cell counts and the measurement of DNA content by the method of Hill and Whatley (15). Mitogenesis was expressed as \(^{3}H\)thymidine-incorporated acid-insoluble counts per 10^6 cells.

To determine the effects of 1% DMF on mitogenesis, cells were plated in standard growth medium which was changed to growth medium containing 1% DMF after 24 hr. This concentration of DMF was maintained in all medium changes throughout the remainder of the experiment.

**RESULTS**

**Effects of DMF on Growth Properties of AKR-MCA Cells.** Several investigators have shown that planar polar reagents reduce the growth rate and saturation density of malignant cells (6, 17). DMF had similar effects on AKR-MCA cells as generation times were increased approximately 75% and saturation density was decreased 10-fold (Table 1). The decrease in saturation density is reflected by DMF-induced alterations in the morphology of AKR-MCA cells (Fig. 1). Cells grown in the presence of 1% DMF lost their transformed morphology and grew as an orderly monolayer of polygonally shaped cells. Moses et al. (23) have shown that AKR-MCA and AKR-2B cells have similar generation times, but AKR-MCA cells have a significantly higher saturation density. We observed a similar relationship between the 2 cell types (Table 1). Normal AKR-2B cells were also affected by treatment with 1% DMF. The generation time of AKR-2B cells was also increased by 75%, and the saturation density was reduced so that both normal AKR-2B cells and malignant AKR-MCA cells had the same generation times and saturation densities in the presence of 1% DMF (Table 1).

Tucker et al. (31) had demonstrated that anchorage-independent growth of AKR-MCA cells was dependent upon cell concentration, while AKR-2B cells did not grow in semisolid medium. When AKR-MCA cells were plated in agarose containing 1% DMF in the standard growth medium, no anchorage-independent growth was observed. These results are in agreement with those of Dexter et al. (6), who showed that anchorage-independent growth of human colonic carcinoma cells was prevented by DMF treatment.

**Effects of DMF on Membrane Antigens of AKR-MCA Cells.** Amido black staining of proteins transferred onto nitrocellulose did not reveal any significant differences in the staining pattern of membrane proteins from AKR-2B, AKR-MCA, or AKR-MCA
cells treated with DMF (Fig. 2A). A sensitive immunochemical approach was necessary in order to bring out subtle and minor changes on membrane components as a result of transformation by MCA and treatment of MCA-transformed cells with DMF. Initial analysis of membrane antigens using the anti-AKR-MCA antiserum revealed several prominent antigenic changes in AKR-MCA cells in comparison to the normal AKR-2B membrane antigens. The expression of several high-molecular-weight antigens (approximate molecular weights ranged from 110,000 to 450,000) was greatly reduced in the AKR-MCA cells; however, exposure to DMF restored the expression of these membrane antigens (Fig. 2B). This antiserum also detected a low-molecular-weight AKR-MCA cell-associated antigen (M, 32,000 to 33,000), the expression of which was barely detectable in the normal AKR cells. Exposure of AKR-MCA cells to DMF suppressed the expression of this particular antigen (Fig. 2B). The elevated expression of a M, 75,000 antigen was found to be associated with AKR-MCA cells in comparison to the AKR-2B cells. The treatment of AKR-MCA cells with DMF resulted in a further enhancement of the expression of this antigen. Two antigens (M, 45,000 and 50,000) that were highly expressed in AKR-2B cells, the expression of which were greatly reduced in AKR-MCA cells, were not restored by DMF treatment of AKR-MCA cells. DMF treatment of AKR-2B cells affected the quantitative electrophoretic pattern of membrane antigens. In other studies, we found that DMF treatment of AKR-2B cells did not alter qualitative electrophoretic patterns, but did lead to an increase of radioiodinated high-molecular-weight cell surface proteins (20).

The reduced expression of the high-molecular-weight antigens in the AKR-MCA cells in comparison to normal AKR-2B or AKR-MCA cells exposed to DMF was also detected by an antiserum raised against the AKR-2B cells. It is conceivable that these high-molecular-weight antigens in the AKR-MCA cells were very immunogenic in the rabbits and were able to elicit antibody responses in the animals. The association of molecular size with immunogenicity has been well documented (5). If the expression of these high-molecular-weight antigens were truly elevated in the normal AKR cells, one would expect to obtain similar results by using an antiserum raised against the normal AKR-2B cells (Fig. 2C). The expression of the high-molecular-weight antigens was greatly reduced and barely detectable in the AKR-MCA cells in comparison to the normal AKR-2B cells; DMF treatment of AKR-MCA cells enhanced the expression of these antigens.

**Effects of 1% DMF on EGF-stimulated Mitogenesis in AKR-MCA Cells.** EGF does not induce mitogenesis of growth-arrested AKR-MCA cells, but is quite effective toward normal AKR-2B cells (23). Mitogenesis of growth-arrested AKR-MCA cells can be induced by nutrient replenishment (23). The differences between the 2 cell types in their response to growth control exerted by exogenous factors provided an opportunity to determine whether the "normalization" of malignant cells by DMF could be demonstrated in the form of restoration of response to normal growth stimuli. The results are summarized in Table 2. As predicted, AKR-MCA cells were not responsive to EGF, since mitogenesis was stimulated equally by nutrient replenishment and nutrient replenishment in the presence of EGF (Table 2A). In contrast, medium replenishment of AKR-2B cells had little effect on mitogenesis (Table 2B, - EGF). When EGF was added to the medium, mitogenesis was stimulated more than 10-fold (Table 2B, + EGF). AKR-MCA cells maintained in the presence of 1%
DMF showed a pattern of response almost identical to that of AKR-2B normal cells and the opposite pattern of response to AKR-MCA cells which were not treated with DMF. Treatment of AKR-2B cells with DMF did not change the response to nutrient replenishment (Table 2D, - EGF). Growth-arrested AKR-MCA cells in 1% DMF did not respond to medium replenishment (Table 2D, - EGF) but did show 10-fold stimulation of mitogenesis upon addition of EGF to the medium (Table 2C, + EGF). DMF treatment of AKR-2B cells resulted in an increased mitogenic response to EGF (Table 2D, + EGF) relative to untreated AKR-2B cells (Table 2B), but this effect was small relative to the 10-fold stimulation of mitogenesis upon the addition of EGF to the medium (Table 2C, + EGF). DMF showed a pattern of response almost identical to that of AKR-MCA cells which were not treated with DMF.

Table 2

<table>
<thead>
<tr>
<th>Conditions</th>
<th>[3H]Thymidine incorporation (cpm/10^6 cells)</th>
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<tbody>
<tr>
<td>A. AKR-MCA control</td>
<td></td>
</tr>
<tr>
<td>+ EGF</td>
<td>15,369</td>
</tr>
<tr>
<td>- EGF</td>
<td>12,443</td>
</tr>
<tr>
<td>B. AKR-2B control</td>
<td></td>
</tr>
<tr>
<td>+ EGF</td>
<td>23,969</td>
</tr>
<tr>
<td>- EGF</td>
<td>1,806</td>
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<tr>
<td>C. AKR-MCA, 1% DMF</td>
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</tr>
<tr>
<td>+ EGF</td>
<td>26,760</td>
</tr>
<tr>
<td>- EGF</td>
<td>2,530</td>
</tr>
<tr>
<td>D. AKR-2B, 1% DMF</td>
<td></td>
</tr>
<tr>
<td>+ EGF</td>
<td>44,645</td>
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<td>- EGF</td>
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DISCUSSION

We have shown that treatment of malignant AKR-MCA cells with 1% DMF resulted in "differentiation or normalization" phenomena similar to those induced by planar polar reagents in other solid-tumor systems in vitro (6, 14, 16, 17). Increased generation time, reduced saturation density, and loss of the ability to grow with anchorage independence were associated with the treatment of AKR-MCA cells with 1% DMF.

We have also demonstrated changes in membrane antigens in association with transformation of AKR-2B cells by MCA and treatment of AKR-MCA cells by DMF. DMF was found to have several effects on the membrane antigens of AKR-MCA cells. There were 2 important changes with respect to transformed cellular effects of DMF on AKR-MCA cells. (a) DMF suppressed the expression of a low-molecular-weight antigen (32 to 33 kilodaltons), the expression of which was associated with MCA transformation; and (b) it restored the expression of several high-molecular-weight antigens (110 to 450 kilodaltons), the expression of which was greatly reduced in association with transformation. However, 2 lower-molecular-weight AKR-2B-associated antigens were not restored by DMF treatment of AKR-MCA cells, and the expression of one AKR-MCA-associated antigen appeared to be enhanced by DMF treatment. These membrane antigen changes were demonstrated by means of a sensitive immunohistochemical procedure. Similar immunohistochemical procedures have been used to detect subtle and minor changes in the chromatin of tumor cells (10, 13, 27).

While planar polar reagents have been shown to induce the expression of specialized molecules associated with differentiation in some tumor systems (11, 14, 16), very little attention has been paid to the possible restoration of normal growth control mechanisms which would be expected to be associated with differentiation or normalization of malignant cells. The AKR culture system offers a good model for these types of studies, since several differences between the growth control of normal AKR-2B cells and their malignant counterparts have been well characterized. Although, under certain conditions, AKR-2B cells can show nutrient-depleted growth arrest (25), under the conditions used for these studies, arrest was accomplished through growth factor depletion (23). AKR-MCA cells do not show growth arrest through growth factor depletion (23). The malignant cells were unresponsive to the addition of exogenous growth factors, because they produce and respond to endogenous growth factors (22, 23).

We have shown that treatment of AKR-MCA cells with DMF restores normal growth control to these cells in a reversible manner. DMF-treated AKR-MCA cells no longer respond to nutrient replenishment, but do respond to exogenously added EGF. DMF-treated AKR-MCA cells now show a "normal" pattern of mitogenic response in that they respond in the same way as normal AKR-2B cells. Differences in response to growth control stimuli between AKR-2B and AKR-MCA cells could be due to differences in the spectrum of endogenous factors produced and/or differences in the abilities of the 2 cell types to respond to the same stimuli. This difference in growth control may be a functional expression of oncogene activation. Recently, extensive sequence homology between platelet-derived growth factor and the oncogene of simian sarcoma virus has been reported (9, 32).

The effect of DMF is not specific for transformed cells, since treatment of AKR-2B cells with DMF resulted in reduction in growth properties of the AKR-2B and AKR-MCA cells. However, the mitogenesis experiments (Table 2) indicated that DMF did not have a toxic effect on the cells. These experiments also indicated that DMF did not change the growth control mechanisms as measured by response to EGF or nutrient replenishment in AKR-2B cells. DMF treatment of the transformed AKR-
MCA cells resulted in a change in the growth control mechanism such that they were similar to the controls in normal cells. The exact mechanism by which DMF induces differentiation is unknown. In some fashion, DMF interferes with the ability of AKR-MCA cells to respond to endogenously produced factors. Possibilities include the reduction of specific factor production by the treated cells, interference with the ability of the cell to interact with its endogenously produced factors, and/or the production of antagonistic factors by treated cells.

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REFERENCES

Restoration of Normal Growth Control and Membrane Antigen Composition in Malignant Cells by \(N,N\)-Dimethylformamide


*Cancer Res* 1984;44:2181-2185.