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Induction of the metastatic phenotype in a mouse tumor model by 5-azacytidine, and characterization of an antigen associated with metastatic activity

(tumorigenicity/non-metastatic/high metastatic subclones/DNA methylation/monoclonal antibodies/isoelectric focusing)

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ABSTRACT **The murine Lewis lung carcinoma is a long-term grafted tumor that, after subcutaneous inoculation, forms metastases to the lungs. Forty-two cell lines were established from a primary tumor site and 40 were established from lung metastatic foci. Cloned sublines were established from the original 82 lines, and 2 sublines among 405 were found to be tumorigenic but not metastatic (T^+/M^-), whereas the remaining 403 sublines were both tumorigenic and metastatic (T^+/M^+). The T^+/M^- phenotype was shown to be stable for >2 yr. However, treatment of the T^+/M^- cell lines for 3 days with 3 μM 5-azacytidine resulted in reexpression of the metastatic phenotype in otherwise stable T^+/M^- lines. Also, 5-azacytidine treatment could result in loss of the metastatic phenotype in lines that had been stable T^+/M^+. The changes in tumorigenic and metastatic phenotypes were not associated with altered immunogenicity of the cells. Monoclonal antibodies were generated against T^+/M^+ cells, and one antibody (M36D3) was found to bind only to T^+/M^+ cells. Reactivity of the antibody was found to co-vary with expression of the metastatic phenotype. The antigen recognized by M36D3 antibody thus seems to be associated with metastatic capability. The antigen was found by two-dimensional gel electrophoretic analysis to be a cellular protein of $M_r \approx 45{,}000$ and pI ≈ 6.7.**

Gene expression is influenced by a variety of biological mechanisms that are only partly known (1–6). It has become increasingly evident, however, that enzymatic methylation of cytosine to 5-methylcytosine (m^5Cyt) is an important gene silencing factor (7–10) in regard to both cellular (11–14) and viral (15–17) genes. This has been substantiated by the fact that demethylation of DNA as obtained by replacement of cytosine with 5-azacytidine (5-azaC) may result in expression of otherwise silent genes in a number of biological systems (18–22), although the biological details still are unknown (5, 6).

Metastatic spread of neoplasms is presumably based on multiple genetic and epigenetic features that are expressed by at least some of the tumor cells (23–26). It is also conceivable that the metastatic phenotype is determined by different gene products in different tumors. It is therefore important to clarify whether metastatic activity can be induced solely by alteration in activity of preexisting genes in nonmetastatic tumor cells.

We report here that the metastatic phenotype can be expressed by otherwise stable nonmetastatic mouse tumor cells after demethylation of DNA of proliferating tumor cells with 5-azaC. Metastatic cells could be identified by a monoclonal antibody (mAb) that recognized an epitope specifically expressed on metastatic cells. The same antibody was found to react with a cellular protein of $M_r \approx 45{,}000$ and pI ≈ 6.7 and two slightly more acidic proteins, which may be modifications of the former.

MATERIALS AND METHODS

Establishment of Cell Lines and Subclones of the Lewis Lung Carcinoma (3LL). Cell lines were established from s.c. growing primary 3LL tumor lesions and from metastatic foci as described in detail elsewhere (27). Biopsies from such tumor lesions were seeded into plastic flasks as explant cultures in RPMI 1640 medium with 15% fetal calf serum. After 3–6 weeks in liquid medium, the cultures were cloned in semisolid 0.3% agar RPMI 1640 medium with 10% fetal calf serum. Clones were harvested from the agar cultures and expanded and maintained in liquid RPMI 1640 medium with 10% fetal calf serum. One cloned culture from each biopsy of either primary tumor lesions or metastatic foci was used to establish subclones. All cell lines were maintained in plastic flasks in RPMI 1640 medium with 10% fetal calf serum/0.3% L-glutamine at 37°C in a humidified atmosphere with 5% CO_2. The cells grew as adherent cultures with cell population doubling times of 20–25 hr in the exponential growth phase. The cells were seeded at a concentration of 2×10^4 cells per cm^2 and were split at a density of $2–4 \times 10^5$ per cm^2; these numbers were used to estimate the number of population doublings (see Table 4).

The original cultures were cloned in 0.3% semisolid agar RPMI 1640 medium with 15% fetal calf serum, and subclones were established from such cultures by harvesting individual colonies with subsequent expansion in liquid medium. Five subclones were established from each culture. All subclones had *in vitro* growth properties comparable to the original cultures.

The cells were treated with 5-azaC by growing the cultures for 3 days in RPMI 1640 with 15% fetal calf serum supplemented with 3 μM 5-azaC, washed twice in phosphate-buffered saline, cultivated for another 10 days in conventional RPMI 1640 medium with 15% fetal calf serum, and then injected s.c. in a dose of $1–5 \times 10^7$ into C57BL/6 mice.

Tumorigenicity and Metastatic Activity. Tumorigenic activity (T^+) was estimated by injection of $0.01–5 \times 10^7$ cells s.c. into 4- to 8-week-old C57BL/6 mice. Three to 10 mice were used to assay for tumorigenic activity of each cell line. It was concluded that a given line was nontumorigenic (T^-) if injection of 5×10^7 cells did not result in tumors up to 4 months after tumor-cell grafting. Thus, 2 of the 208 sublines from cultures of primary tumor lesions did not result in tumors on s.c. injection into 20 mice (10 mice for each line).

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Abbreviations: 5-azaC, 5-azacytidine; FACS, fluorescence-activated cell sorter; IEF, isoelectric focusing; 3LL, Lewis lung carcinoma; mAb, monoclonal antibody; m^5Cyt, 5-methylcytosine; NEPHGE, nonequilibrium pH gel electrophoresis.

Metastatic activity (M^+) was determined on s.c. injection of 0.01–5 × 10^7 tumor cells. Mice were normally killed 4–6 weeks after tumor inoculation, and the number of lung metastases was enumerated under a stereomicroscope. The number of lung metastases was determined in 5–10 mice per tumor cell line. M^- cell lines were defined as cell lines that did not result in visible metastases in 10–15 mice up to 4 months after s.c. inoculation of 5 × 10^7 tumor cells.

Athymic nude C57BL/6 mice were obtained from Bomholtgård (Ry, Denmark) and were like conventional C57BL/6 mice challenged with the various types of 3LL cell lines.

mAb. A mAb [M36D3 $IgG_{2a}(\kappa)$] was generated by fusion of the X63.Ag8.6.5.3 mouse myeloma cells with spleen cells from BALB/c mice hyperimmunized with highly metastatic 3LL cells. The Ig product of the resulting hybridomas was analyzed for cell specificity by cell-binding ELISA and by a fluorescence-activated cell sorter (FACS) (28). Hybrids secreting Ig with strong binding to T^+/M^+ cells and low or no binding to T^+/M^- were cloned and expanded as ascites tumors. The mAbs were then tested by ELISA and FACS for binding to lymphocytes, monocytes, fibroblasts, bone marrow cells, and erythrocytes from C57BL/6 mice. Clone M36D3 secreted an antibody that only bound to T^+/M^+ cells in the panel.

Characterization of Antigen Reacting with M36D3 mAb. The cellular proteins bound to M36D3 were visualized by autoradiography after separation of immune complexes by two-dimensional gel electrophoresis (29, 30). The cells were seeded in microtiter plates (Falcon no. 3040) at a density of 1000–3000 cells per well in 200 μl of RPMI 1640 medium. Two days later, the cells were labeled overnight in RPMI 1640 medium with 10% dialyzed fetal calf serum and low methionine concentration (<2 μg/ml) but supplemented with 250 μCi of [^{35}S]methionine (1 Ci = 37 GBq) (30). The labeled cells were scraped from the bottom of the well in 100 mM NaCl/20 mM Tris·HCl, pH 7.6/1 mM EDTA/0.2% Triton X-100 containing 1 mM phenylmethylsulfonyl fluoride. The cells were lysed by repeated pipetting through the tip of a Gilson pipette and stored at −80°C. The lysed cell extract was clarified by centrifugation at 20,000 × *g* for 1.5 min prior to immunoprecipitation, and ≈10^6 cpm in solubilized protein was then incubated with the mAb [M36D3 or as control Lyt-1 (31), which binds specifically to mouse T lymphocytes] for 30 min at 4°C.

The immune complexes were subsequently incubated for 20 min at 4°C with protein A Sepharose CL-4B or as control with inactivated Sepharose CL-4B. The absorbed immune complexes were pelleted and washed four times as described (29). The pellet was solubilized in lysis buffer containing 1% $NaDodSO_4$, heated for 3 min at 70°C, and polypeptides were separated by two-dimensional gel electrophoresis (30). The gels were treated with 2,5-diphenyloxazole, dried, and fluorography was carried out for 2 months at −80°C.

RESULTS

Cell lines were established from primary s.c. growing 3LL tumor lesions and from individual lung metastases (Table 1). Five cloned sublines of each line were established, and the sublines were subsequently analyzed for tumorigenicity (T^+) and metastatic (M^+) activity in female C57BL/6 mice. Six of the sublines were highly metastatic (>50 metastases per lung per 10^5 cells injected s.c.) and two were only tumorigenic (0 metastases per lung per 0.01–5 × 10^7 cells injected s.c.; when moribund, all animals were examined for lung metastases). The T and M features of the six metastatic sublines were tested every 2–3 months and have been stable >2 yr. In addition, we attempted to generate murine mAbs binding to antigens associated with expression of the malignant phenotype. One antibody M36D3 with such specificity was obtained (Table 1).

Forty-five randomly selected T^+/M^+ lines (23 from primary tumor lesion and 22 from metastatic foci) were treated with 5-azaC. Two lines lost both their *in vivo* tumorigenic and metastatic activity but retained their *in vitro* growth capability, 17 lost the metastatic phenotype, and 26 clones were unchanged after 5-azaC treatment. The same T and M properties were observed for all clones on inoculation into athymic nude C57BL/6 mice (data not shown). Expression of H-2 antigens was unaltered in all lines as tested by FACS with a polyclonal allogenic antibody against $H\text{-}2^b$ (data not shown). Loss of metastatic activity co-varied with loss of expression of the epitope for the M36D3 antibody as detected by FACS (Table 2).

5-azaC treatment of 30 T^+/M^- subclones established from the original 2 T^+/M^- clones resulted in expression of metastatic activity in 11 subclones, 3 clones became T^-/M^-, and 16 clones were unchanged T^+/M^-. Again, the same T^- and M^- features were observed on inoculation into athymic nude C57BL/6 mice. However, whereas the original 3LL murine tumor selectively metastasizes to the lungs, the clones expressing metastatic behavior only after 5-azaC treatment formed metastases not only in the lungs but also in some cases in the liver and in the spleen. It is noteworthy that liver and spleen metastases were not observed after 5-azaC treatment of cells that originally expressed the metastatic phenotype prior to treatment (Table 3). All cell lines were tested again for tumorigenic and metastatic activity 40, 120, and 200 cell generations after 5-azaC treatment (Table 4). Most lines reversed during the first 120 cell generations to the T and M phenotype they had prior to 5-azaC treatment. However, 5 sublines retained the T^+/M^+ phenotype they obtained by 5-azaC treatment. All 5 lines metastasized and bound the M36D3 mAb, whereas none of the lines that reverted from T^+/M^+ to T^+M^- expressed the epitope for M36D3 (Table 4).

Immunization of conventional C57BL/6 mice with T^+/M^+, T^+/M^-, or T^-/M^- cells (3 injected s.c., 5 × 10^6 cells per injection on days 0, 14, and 21) did not in any clone result in

Table 1. Tumorigenic, metastatic, and antigenic properties of cell lines established from primary tumors and lung metastatic lesions of the 3LL murine carcinoma

Origin of cell line (*n*)	No. of subclones	No. of T^+ lines/ total no. of cell lines	No. of T^+/M^- cell lines/total no. of lines	No. of low to medium M^+ lines*/total no. of cell lines	No. of high M^+ lines†/total no. of lines	No. of M36D3 lines‡/ total no. of cell lines
Primary tumor (42)	208	208/208	2/208	206/208	0/208	206/208
Lung metastatic lesions (40)	197	197/197	0/197	191/197	6/197	197/197

*5–50 metastases per 10^5 cells, s.c.
†>50 metastases per 10^5 cells, s.c.
‡M36D3 binding was determined by FACS analysis. Tumor cells were trypsinized in plastic flasks, cultivated in RPMI 1640 with fetal calf serum in a flask on a rocking platform (to prevent reattachment, but to allow restoration of cell surface attributes) for 24 hr, and stained in a two-step procedure with M36D3, using fluorescein isothiocyanate-conjugated rabbit-anti-mouse Ig as second-step antibody. A shift of 25 channels of the fluorescence was required to consider a sample positive.

generation of cytotoxic antibodies to any of the 3LL clones (data not shown), indicating that the 5-azaC treatment did not result in increased immunogenicity in C57BL/6 mice.

Four types of cells were analyzed by immunoprecipitation with the M36D3 mAb: T^+/M^+, T^+/M^-, and the same cells 2 weeks after 5-azaC treatment. The T^+/M^- cells were converted by 5-azaC into T^+/M^+. The cells were labeled overnight with [^{35}S]methionine as described. Immune complexes precipitated with the M36D3 mAb or Lyt-1 (control) were analyzed by two-dimensional gel electrophoresis according to size and charge (29, 30), half being used for isoelectric focusing (IEF) of acidic proteins and the other half were used for nonequilibrium separation (NEPHGE) of alkaline proteins.

The precipitate with M36D3 and protein A Sepharose from T^+/M^+ cells consistently contained one major precipitate of $M_r \approx 45{,}000$ and pI ≈ 6.7 (Fig. 1, large arrow) that migrates near the origin of both IEF and NEPHGE gels in accordance with a certain overlap near neutral pH of these two types of gels. Close to the acidic side of the major spot, two polypeptides could be seen in small amounts (small arrows). These most likely represent charge modifications of the major component. A few other faint spots with an intensity no stronger than the above-mentioned minor spots could be seen in several gels (arrowheads); these were due to nonspecific binding to Sepharose (see below).

The major component of the immunoprecipitate and the two acidic neighboring spots were detected in extracts from the T^+/M^+ cells, irrespective of prior treatment with 5-azaC and perhaps more intense in extracts from cells treated with 5-azaC. No major polypeptides were precipitated from T^+/M^- cells regardless of any pretreatment with 5-azaC.

Three faint spots corresponding to $M_r \approx 59{,}000$, pI ≈ 5.8; $M_r \approx 63{,}000$, pI > 7.2; and $M_r \approx 40{,}000$, pI > 8.5 were seen in many autoradiograms (Fig. 1, arrowheads). These spots were due to unspecific precipitation with the Sepharose beads, because they were present in precipitates from all four cell types, regardless of type of mAb or whether the Sepharose contained protein A.

DNA methylation of the lines was also measured prior to 5-azaC treatment and at various time intervals afterward (Table 5). Most 5-azaC-treated clones were hypomethylated compared to nontreated cells. However, two of the five clones that gained and maintained metastatic activity were not hypomethylated.

DISCUSSION

The genetic basis for tumorigenicity is currently studied by transforming preneoplastic mouse cells by DNA fragments of malignant cells of both animal and human origin (33–36).

Table 2. Effect of 5-azaC on the tumorigenic and metastatic capability of 45 T^+/M^+ subclones

No. of subclones with *in vitro* growth potential/ total no. of clones	No. of T^+ clones	No. of clones metastazising to lungs*	No. of clones metastazising to other organs	No. of clones binding the M36D3 mAb†
45/45	43	26	0	26

The cell cultures were grown for 3 days in RPMI 1640 medium with 15% fetal calf serum, supplemented with 3 μM 5-azaC, grown for another 10 days in conventional RPMI 1640 medium with 15% fetal calf serum, and then injected (0.01–5 × 10^7 cells, s.c.) into C57BL/6 mice.

*The number of metastases ranged from 20 to 40 per lung 4–6 weeks after s.c. injection of 10^5 cells. Loss of metastatic activity (M^-) was defined as lack of metastatic foci in the lungs, liver, and spleen up to 4 months after s.c. inoculation of 1–5 × 10^7 tumor cells.

†Determined as described in Table 1.

Table 3. Effects of 5-azaC on 30 tumorigenic but not metastatic 3LL subclones

No. of T^+ clones/total no. of clones	No. of M^+ clones metastazising to the lungs*	No. of M^+ clones metastazising to other organs (spleen and liver)†	No. of clones binding M36D3 mAb	
			T^+/M^-	T^+/M^+
27/30	11	8	0/16	11/11

*The number of metastatic foci resulting from T^+/M^+ cell lines that have converted from T^+/M^- by 5-azaC treatment was counted 4–6 weeks after s.c. injection of 10^5 cells. The number of metastatic foci ranged from 9 to 16 per lung, which was one-half to one-third the number obtained by conventional 3LL cells.

†The number of metastatic lesions on the surface of the liver and the spleen was counted under a stereomicroscope at the same time the lung metastases were enumerated. All clones that metastazised outside the lungs formed metastatic lesions in both liver and spleen.

However, these studies have not yet enabled us to analyze the gene(s) that mediate metastatic capability. It is possible that some of the genes required for tumorigenicity also are necessary for metastatic activity, but the fact that we have been able to establish tumorigenic, nonmetastatic cell lines (in contrast to most other studies that have used high and low metastatic cell lines) in the 3LL system shows that at least a part of the genetic basis for metastatic activity differs from that for tumorigenicity. Our tumor model should, therefore, enable us to further identify these genes.

5-azaC is incorporated at multiple sites into the DNA and may also lead to demethylation at the level of the hemimethylases (37). It is, therefore, likely that a multitude of genes irrelevant for metastatic activity are demethylated. Consequently, the total amount of m^5Cyt cannot be used to evaluate the role of m^5Cyt in regulation of expression of genes relevant for metastatic activity. A number of cell lines reversed after a variable number of cell divisions to the phenotype observed prior to 5-azaC treatment. The enzymatic DNA methylation pattern in this system was, therefore, not strictly heritable, in line with reports in other cell systems (14, 38, 39).

5-azaC is not (or only negligibly) mutagenic (40) in the doses used in this study. This was recently substantiated by the observation (unpublished data) that 5-azaC in doses <8 μM did not induce any increase in the number of ouabain- or 6-thioguanine-resistant cell variants of the 3LL line, a human B-lymphoma line, a human squamous lung carcinoma line,

Table 4. Tumorigenic and metastatic activity of 3LL sublines at various time intervals after they were altered in their T/M features by 5-azaC

	No. of population doublings after 5-azaC treatment			
	≈10	≈40	≈120	≈200
No. of T^+/M^+ clones that obtained M^+ properties after 5-azaC treatment	11/11	9/11	5/11	5/11
No. of the same 11 T^+/M^+ clones binding the M36D3 mAb	11/11	9/11	5/11	5/11
No. of lines becoming T^+/M^+ after having converted to only T^+/M^- from T^+/M^+ after 5-azaC treatment	0/17	3/17	16/17	17/17
No. of cultures binding the M36D3 mAb	0/17	3/17	16/17	17/17

Population doublings were estimated from the number of cell passages times the inverse split ratio.

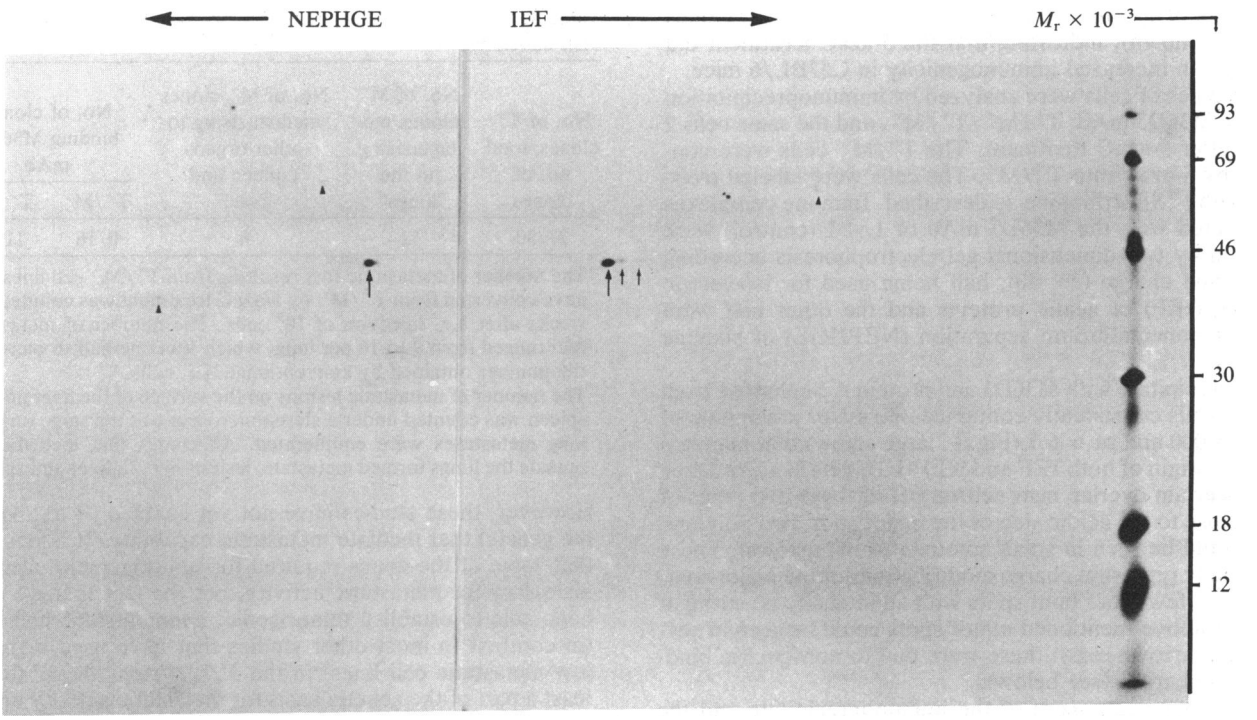

FIG. 1. Immunoprecipitation with M36D3 mAb of solubilized proteins from Lewis lung cells with T^+/M^+ phenotype and analysis by two-dimensional gel electrophoresis. Alkaline proteins are shown on the left, and acidic proteins are on the right. Large arrows indicate the major protein (M_r, ≈45,000; pI, ≈6.7) specifically precipitated by the M36D3 antibody and shown in both NEPHGE and IEF gels. The two small arrows indicate minor amounts of protein precipitated by the same mAb. The three arrowheads indicate minor amounts of proteins unspecifically precipitated bound to Sepharose beads. Fluorography was carried out for 60 days at −80°C with Kodak XAR 5 film.

and a human myeloma cell line. The results are therefore best explained as a result of 5-azaC-mediated DNA demethylation, leading to increased expression of genes that in the nonmetastatic clones have been silent or have had very low activity since the cell lines were established (>2 yr ago). Thus, stable nonmetastatic tumor cells contain gene(s) that appropriately expressed can result in metastatic behavior. It seems of ample importance to identify these genes, because they, together with genes encoding for features leading to tumorigenic properties, probably are crucial for malignancy (41) in the 3LL tumor.

One of the gene products associated with the metastatic process could be identified with a mAb (M36D3). The antibody bound to a protein of $M_r \approx 45{,}000$ and pI ≈ 6.7, as measured under reduced denaturing conditions. The protein was observed as the major precipitate from extracts of T^+/M^+ cells with M36D3 (Fig. 1) as well as from extracts of 5-azaC-treated cells (data not shown). A low amount of this protein seemed to be present in the T^+/M^+ cells in two minor precipitates with the same M_r but with one or two extra acidic groups (Fig. 1, small arrows). 5-azaC treatment of T^+/M^+ cells did not significantly increase the intensity of this major precipitate, but a small relative increase cannot be ruled out. The protein of $M_r \approx 45{,}000$ and pI 6.5–6.8 was barely detectable in precipitates from T^+/M^- cells on the two-dimensional gels (and not detectable by FACS analysis), indicating a greatly decreased amount relative to T^+/M^+ cells. This is in agreement with the assumption that the anti-

Table 5. DNA methylation in subclones of 3LL at various time periods after 5-azaC treatment

Cell lines	% m^5Cyt of total cytosine		
	Prior to 5-azaC	After ≈10 generations	After >200 generations
32 lines from primary tumor and 28 lines from metastases	4.8 ± 0.3	ND	ND
16 T^+/M^+ lines that converted to T^+/M^- after 5-azaC treatment	4.6 ± 0.5	1.6 ± 1.2	4.1 ± 0.6
14 T^+/M^+ lines that remained T^+/M^+ after 5-azaC treatment	4.5 ± 0.2	1.3 ± 0.7	3.8 ± 0.7
11 T^+/M^- lines that became T^+/M^+ after 5-azaC treatment	4.9 ± 0.2	1.6 ± 0.5	3.6 ± 1.2
12 T^+/M^- lines that remained T^+/M^- lines after 5-azaC treatment	4.7 ± 0.4	1.1 ± 0.6	4.4 ± 0.6
5 T^+/M^- lines that gained stable T^+/M^+ features after 5-azaC treatment			
Line: 1	4.7 ± 0.2	<1.0	3.9 ± 0.3
2	4.8 ± 0.3	1.7 ± 0.1	1.2 ± 0.2
3	4.6 ± 0.2	1.3 ± 0.2	4.3 ± 0.2
4	4.8 ± 0.3	<1.0	1.8 ± 0.3
5	4.6 ± 0.2	<1.0	1.9 ± 0.2

DNA methylation was estimated as the percentage of methylated cytosines in newly replicated DNA. Cell cultures were supplemented with 120 nCi of deoxy[^{14}C]cytidine (specific activity, 460 Ci/mol; Amersham) per ml of culture medium. DNA was hydrolyzed into its bases in 96% formic acid for 60 min at 175°C. The bases were separated by thin-layer chromatography (32) and the percentage of enzymatic methylation was calculated as 100% × m^5Cyt (cpm)/Cyt (cpm) + m^5Cyt (cpm). ND, not determined.

gen is closely associated with metastatic activity.

From the duration of exposure during fluorography (2 months) it can be estimated that the major precipitate constitutes <0.01% of the labeled proteins in the solubilized cellular proteins. β_2-microglobulin was not observed in the immunoprecipitates, and because nonmetastatic cells express high amounts of H-2 antigens it can be ruled out that the M36D3 antibody recognizes an H-2 epitope.

Two-dimensional analysis of T^+/M^- cells that were converted to T^+/M^+ cells after 5-azaC treatment failed to reveal increased amounts of the protein recognized by M36D3, despite the fact that FACS analysis showed increased expression of the epitope detected by M36D3, which was quantitated by the shift in channel numbers for mean fluorescence intensity. This is conceivably due to an alteration in presentation of the antigen as a result of hypomethylation of the cells.

It is also of interest that loss of T and/or M features was not a result of increased immunogenicity of some subclones, as was otherwise observed recently by Kerbel *et al.* (39) in murine mammary carcinomas, because the T and M features of the various lines were the same in conventional mice and in athymic nude mice. In addition, no signs of increased immunogenicity of T^+/M^- or T^-/M^- were observed on immunization of conventional C57BL/6 mice.

Intratumoral phenotypic diversity of malignant neoplasms has been shown with respect to a number of biological features, including those for metastasis (23). The biological basis for this diversity is unknown, but it can be assumed to be a result of a number of genetic and epigenetic mechanisms. The present results show that the phenotypic diversity, at least in part, can be obtained solely by alteration of the enzymatic DNA methylation pattern and support the idea that the phenotypic features of a tumor cell population are continuously subject to change (42–44).

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