SELECTION OF STRONGLY IMMUNOGENIC "TUM-" VARIANTS FROM TUMORS AT HIGH FREQUENCY USING 5-AZACYTIDINE

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Treatment of normally tumorigenic murine tumor cell lines in vitro with chemical mutagens followed by cloning of the surviving cells, results in the selection, at extraordinarily high frequencies (anywhere from <1% to >90%), of clones unable to grow progressively in normal syngeneic mice (1-7). Such clones, which are phenotypically stable in culture over a period of several weeks or months, have been designated by Boon and his colleagues (1-6) as "tum-" (non-tumorigenic in normal hosts). They have been derived using such standard mutagens as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and ethyl methanesulfonate (EMS), from at least eight different murine tumors of viral, chemical, or spontaneous origin (1-7). Because the tum- clones will grow readily in highly immunosuppressed recipients, e.g., X-irradiated or nude mice (1-8), the tum- phenotype appears to have an underlying immunological basis, a view confirmed by detailed in vitro studies of T cell-mediated cytotoxicity (CMC).

These studies have also shown that each tum- clone derived from a particular mutagenized parent line possesses an individual tumor-specific antigen distinct from the new antigen found on any other tum- clone, i.e., there is a startling degree of tumor antigen polymorphism (1-7). Of considerable interest is that this holds true even for totally non-immunogenic tumors of spontaneous origin (5-7) and as such, has very broad and important implications for the field of tumor immunology, especially in relation to the controversies surrounding the antigenic and immunologic status of neoplastic cells (9-11). Nevertheless, very little is known about how mutagens can cause such drastic and heritable changes in the behavioral properties of tumors in vivo, and at such high frequencies.

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1 Abbreviations used in this paper: C, cytosine, CMC, T cell-mediated cytotoxicity; CTL, cytotoxic T lymphocyte; EMS, ethyl methanesulfonate; HPLC, high performance liquid chromatography; MC, 5-methylcytosine; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; tum-, non-tumorigenic in normal hosts.
However, because these frequency rates are many orders of magnitude greater than that predicted by classic genetic (point) mutational events, there is the distinct possibility that an "epigenetic" mechanism is involved. By "epigenetic" we mean a heritable phenotypic change brought about by a mechanism that does not involve a change in the actual sequence of nucleotides in DNA. Such a change can be brought about by "DNA hypomethylation."

Many investigators (reviewed in references 12 and 13) have reported that a drop in the level of 5-methylcytosine in DNA can result in expression of genes that were previously "silent." Such DNA hypomethylation can be achieved by the treatment of cells with the DNA hypomethylating agent 5-azacytidine, an analogue of the DNA base, cytosine. This can result in high frequency phenotypic alterations (in the order of 10%) as a result of alteration in gene expression. Furthermore, the altered methylation patterns and levels can be somatically inherited (12, 13), though not with perfect fidelity (14), thereby raising the distinct possibility that "methylation changes can masquerade as mutations" (13). Finally, a number of investigators have reported that many mutagens (including EMS and MNNG) and carcinogens can themselves cause hypomethylation of DNA (13, 15-17). It therefore seemed reasonable to hypothesize that hypomethylation of DNA could explain the high frequency at which the tum- clones are generated after mutagen treatment. We therefore decided to test the tumorigenic and immunologic properties of tumor cell clones that had been pretreated with EMS or 5-azacytidine. The results demonstrate that 5-azacytidine can cause virtually the same changes as EMS, despite an absence of any significant mutagenic effect in the treated cells.

Materials and Methods

Animals. DBA/2 and A/J strain mice 5-6 wk old, were purchased from The Jackson Laboratory, Bar Harbor, ME. BALB/c-nu/nu mouse breeding pairs were originally obtained from the National Institutes of Health (NIH) and are currently maintained in a nude mouse colony established in the research facilities of the Long Beach Veterans Administration Hospital. In all experiments aimed at assessing the growth potential of tum- variants, both nu/nu and nu/+ heterozygous littermates were challenged with tumor.

Tumors. The origin and characteristics of the MDAY-D2 tumor have been described in detail elsewhere (18, 19). The P815 mastocytoma was obtained from Dr. R. Herberman (NIH). The TA3 mammary adenocarcinoma was obtained through Dr. Joseph G. Mayo, NIH, and Dr. Arthur Bodgen, Mason Research Institute Tumor Bank, Worcester, MA. Tumor cells were maintained in culture or by weekly intraperitoneal passage. All cell lines were routinely tested to assure that they were free of Mycoplasma by Dr. Eric Stanbridge (Department of Microbiology, UCLA/Irvine). Only Mycoplasma-free cell lines were used in these studies.

Treatment of Cells with Mutagens. Treatment of tumor cells with MNNG was performed according to the methods of Boon et al. (1-6). Cells (3 × 10⁶ in 5 ml) were exposed to MNNG at 3 μg/ml for 60 min. The cells were washed and placed in culture. Treatment with EMS was according to the method of Gillin et al. (20). 5 ml of tumor cells at 6 × 10⁶ cells/ml were placed in small, sterile petri dishes and allowed to equilibrate to a 5% CO₂ atmosphere in RPMI without fetal calf serum. After 1 h, 2.5 μl (360 μg/ml) of EMS was added, and the cells were allowed to incubate for 2 h, after which they were centrifuged and incubated in RPMI medium with 10% fetal calf serum. Under these conditions, cell viability is reduced to ~30% after 24 h. This is consistent with the findings of others (1-6).
Treatment of Cells with 5-Azacytidine. Treatment with 5-azacytidine was performed according to the method of P. Jones (personal communication). 5-Azacytidine (final concentration: 3 μM) was added to 5 ml of cells at 6 × 10^6 cells/ml. The cells were washed after 24 h incubation and allowed to re-grow for 1 wk before cloning. Cell viabilities after 5-azacytidine treatment generally exceeded 90%. After cloning, cells from individual clones were injected subcutaneously (5 × 10^5 cells) into syngeneic mouse hosts. Clones that failed to grow were tested again, i.e., they were reinjected into normal syngeneic hosts twice more to assess the stability of the tum- phenotype. In addition, individual tum- clones were injected into nude mice and each was also assessed for its ability to protect against a parent tumor challenge and to induce a cytotoxic T lymphocyte (CTL) response.

Assessment of T Cell Cytotoxicity. A standard method to induce secondary CTL responses was used which involves in vitro restimulation (7, 18). Three DBA/2 or A/J strain mice were given subcutaneous injections of 5 × 10^5 cells from each of the P815-5-azacytidine–induced tum- clones (J or D) or TA3-5-azacytidine–induced tum- clones (K or P), respectively. 14 d later, the spleens were removed, pooled, and restimulated in vitro with mitomycin C–treated cells identical to the initial priming clone. 5 d later, the cells were harvested and used as effectors against different targets, in an ^111Indium release assay (18).

Assessment of Mutagenic Potential of EMS vs. 5-Azacytidine. Cells treated with EMS or 5-azacytidine above were assessed for the number of induced ouabain-resistant (Oua^r) 6-thioguanine-resistant (6-TG^r) or 2-deoxy-D-galactose-resistant (2-D-gal^r) colonies as follows. For each experiment, 4.8 × 10^6 tumor cells from various tumor lines were plated into 96-well microtiter plates (each well containing 10^4 cells in 200 μl of culture medium) in the presence of either 3 mM ouabain, 10^-4 M 6-thioguanine, or 3 mM 2-deoxygalactose. The number of drug-resistant colonies was determined 11–14 d later.

Assessment of DNA 5-Methylcytosine Content by High Performance Liquid Chromatography (HPLC). The HPLC method used to quantitate 5-methylcytosine was based on that described by Flateau et al. (21). Cells were labeled with [3-H]-uridine by incubating 2 × 10^5 cells/ml with 2 μCi/ml uridine. A Beckman HPLC system with a Altex ULTRASIL-CX column was used with isocratic elution using 0.085 M KH_2PO_4, pH 2.5 containing 5% vol/vol MeOH at a flow rate of 2.5/min. The elution of the bases was measured using known standards. The fractions containing the cytosine (C) and 5-methylcytosine (MC) were assayed for radioactivity by scintillation counting. Percentage MC (21) was calculated as follows: % MC = cpm (MC)/cpm (C) × 100%.

Results

Frequency of Selection of Tum- Clones. Three different tumors treated with 5-azacytidine were cloned and assessed for the tum- phenotype, and the results compared to those obtained using conventional mutagens (MNNG or EMS) with the same tumors. The frequency of selection of tum- variants derived after 5-azacytidine treatment (and compared to frequencies seen after mutagen treatment) is shown in Table I. 15 randomly selected tum- variants of TA3 were reinjected into A/J mice or BALB/c nude mice on two subsequent occasions. 14 of these clones retained their tum- phenotype. Of these 14 clones, 4 were injected an additional four times over a six-month period and remained stable for their tum- phenotypes. Of these four, however, only one has remained tum- after nine months in culture. No tum- MDAY-D2 clones were detected after 5-azacytidine treatment. This is identical to what was observed when the same tumor was treated once with EMS or MNNG as shown in Table I, and as described previously. Tum- variants from MDAY-D2 could be generated, but only at low frequency after a double (sequential) treatment with the mutagen (7). Double (sequential) treatment with 5-azacytidine did not generate any
| Tumor line tested          | Frequency of tum- clones after treatment in vitro with* | Growth of tum- clones in nude mice |
|---------------------------|---------------------------------------------------------|----------------------------------|
|                           | EMS       | MNNG       | 5-Azacytidine |                                                          |
| TA3 Adenocarcinoma        | 26/28     | 23/24      | 108/124       | 8/8                                                      |
|                           | (>90%)    | (>90%)     | (87%)         |                                                          |
| MDAY-D2 (undifferentiated | 0/180     | 3/24       | 0/124         | 3/3                                                      |
| tumor)                    | (0%)      | (12.5%)    | (0%)          |                                                          |
| P815 Mastocytoma          | 0/40      | 0/40       | 4/24          | 4/4                                                      |
|                           | (0%)      | (0%)       | (16.7%)       |                                                          |

* Tumor cells were treated with mutagens (EMS or MNNG) or 5-azacytidine, and cloned by limiting dilution. 5 x 10^6 cells from individual clones were injected subcutaneously, into syngeneic mice and their growth assessed. Randomly selected tum- clones were also injected subcutaneously into nude mice and observed for growth.

MDAY-D2 tum- variants. All four tum- P815 clones that were isolated after 5-azacytidine treatment retained their tum- phenotype after three months in culture.

**Immunogenicity of Tum- Clones Derived after 5-Azacytidine Treatment.** In order to demonstrate that the failure of tum- clones to grow was due to an immunological change, eight of the TA3 and four of the P815 5-azacytidine-induced tum- clones were injected into T cell-deficient athymic (nude) mice. All of these clones, which failed to grow in syngeneic normal animals, grew progressively and killed the nude mouse host. Furthermore, all TA3 5-azacytidine-induced tum- clones (N = 23) and P815 5-azacytidine-induced tum- clones (N = 4) were able to protect syngeneic hosts against challenge with the parent tumor. Thus A/J strain mice preimmunized with individual TA3 5-azacytidine-induced tum- clones and subsequently challenged with ordinarily lethal doses of TA3 tumor cells have survived indefinitely (>10 months) without evidence of tumor recurrence. Furthermore, this protection was specific in that animals immunized with a 5-azacytidine-induced tum- clones of TA3, were not protected against a subsequent challenge with an unrelated A strain tumor, i.e., the H6TBJ-750 hepatoma (data not shown).

All the 5-azacytidine–induced tum- variants tested (for TA3 N = 6; for P815 N = 4) were able to induce CTL response after appropriate secondary stimulation (Table II). While this response was sometimes less than that seen with EMS- or MNNG-derived tum- clones (7), it was far greater than that seen in animals injected with the parent tumor (Table II). In addition, as noted above, this CTL response was sufficient for the specific protection against parent tumor challenge in vivo.

**Evidence That 5-Azacytidine Is Not Mutagenic in Murine Tumor Cells.** It is evident, from these experiments, that 5-azacytidine, which has been shown to be non-mutagenic, or very poorly so, in non-neoplastic mouse C3H 10T½ cells or Chinese hamster lung (V79) cells (22) is as effective in selecting immunogenic tum- variants as EMS or MNNG (or as ineffective, in the case of MDAY-D2). Because the mutagenic effects of 5-azacytidine on mouse tumor lines have not yet been assessed, we tested its effect on the frequency of drug-resistant mutants.
### Table II

**Cytotoxic T Cell Responses Generated by 5-Azacytidine Selected Tum" Clones**

| Mice immunized with: | Targets | % Cytotoxicity at 50/1 E/T Ratio |
|----------------------|---------|----------------------------------|
| TA3-clone K          | TA3-clone K | 32 ± 1.4                        |
| TA3-clone K          | TA3-clone P | 38 ± 2.1                        |
| TA3-clone K          | TA3 (parent)| 27 ± 1.6                        |
| TA3-clone P          | TA3-clone P | 37 ± 0.8                        |
| TA3-clone P          | TA3-clone K | 39 ± 0.6                        |
| TA3-clone P          | TA3-(parent)| 28 ± 1.2                        |
| TA3 (parent)         | TA3-(parent)| <5%                             |
| TA3 (parent)         | TA3-clone K | 0                               |
| TA3 (parent)         | TA3-clone P | 0                               |
| P815-clone J         | P815-clone J | 100                             |
| P815-clone J         | P815-clone D | 90 ± 2.4                        |
| P815-clone J         | P815-(parent)| 85 ± 1.6                        |
| P815-clone D         | P815-clone D | 83 ± 3.1                        |
| P815-clone D         | P815-clone J | 56 ± 0.8                        |
| P815-clone D         | P815 (parent)| 73 ± 1.4                        |
| P815 (parent)        | P815 (parent)| <5%                             |

* Two tum- clones from TA3 and P815 were injected subcutaneously into syngeneic hosts from which the spleens were removed 14 d later. Spleen cells were restimulated with mitomycin C-treated tumor cells identical to the priming clone. 5 d later the spleen cells were used as effectors against various targets. Control mice were immunized with cells from the untreated parent tumor.

### Table III

**Frequency of Oua^a, 6-TG^, and 2-D-gal^ Mutants Obtained after Treatment of MDAY-D2 or TA3 Tumor Cells In Vitro with 5-Azacytidine or EMS**

| Cell line | Treatment*          | No. of drug^a colonies x 10^4 | 3 mM Ouabain | 10^4 M 6-Thioguanine | 3 mM 2-Deoxygalactose |
|-----------|---------------------|-------------------------------|--------------|---------------------|-----------------------|
| MDAY-D2   | No treatment        | 0                             | 0            | 0                   |
| MDAY-D2   | 3 μM 5-azacytidine  | 0.2                           | 8            | 0.3                 |
| MDAY-D2   | 360 μg/ml EMS       | 29                            | 70           | 10                  |
| TA3       | No treatment        | 0                             | 0            | ND                  |
| TA3       | 3 μM 5-azacytidine  | 1                             | 0            | ND                  |
| TA3       | 360 μg/ml EMS       | 15                            | 1.3          | ND                  |

*Treatments were for 24 h, in complete medium and cells were plated 4–6 d later. Results are average of two experiments except for the results recorded for the number of 6-TG^ and 2-D-gal^ MDAY-D2 colonies obtained after EMS treatment, which represent one experiment only. ND, not done.

in MDAY-D2 or TA3 cells and compared the results to those obtained after EMS treatment. As shown in Table III, EMS, as expected, produced a significant
increase in Oua\textsuperscript{R}, 6-TG\textsuperscript{r} or 2-D-gal\textsuperscript{r} colonies. However, 5-azacytidine had no effect, or only a negligible one, in all cases. Thus, the results confirm those of Landolph and Jones (22), and strongly imply that 5-azacytidine is a poor inducer of various types of point mutations in mouse tumor cell lines.

Effects of 5-Azacytidine or EMS on the Levels of 5-Methylcytosine in DNA of TA3 or MDAY-D2 Cells. As shown in Table IV, we have made some preliminary analyses, using HPLC, of the levels of 5-methylcytosine in EMS or 5-azacytidine-treated tumor cells. The results show that a decrease in methylcytosine content was observed in 5-azacytidine-treated cells (of \(\sim 15\%\)), as expected, though it was not as great as we had anticipated based on the results of others (where decreases of about 50\% are often observed, e.g., references 23–25). When EMS-treated cells were examined, no decrease was detected in MDAY-D2 cells (from which tum\textsuperscript{-} variants are not derived after one-step EMS treatment). A small decline was noted in the EMS-treated TA3 cells; however, this was not assessed to be statistically significant.

Discussion

The purpose of these studies was to determine whether the treatment of murine tumor cells with 5-azacytidine, like EMS, would result in the derivation of clones at high frequency unable to form tumors in normal syngeneic hosts. Such selections had previously been reported by Boon and his colleagues (1–6) after treatment of murine tumors with the alkylating agent MNNG, and by ourselves, using EMS (7). While the availability of such so-called tum\textsuperscript{-} variants is of considerable interest in itself, the expression of unique tumor antigens by each tum\textsuperscript{-} clone (1–6) in conjunction with their derivation at such high frequencies raises several important immunologic and genetic questions (11). One of the most obvious of these relates to the genetic mechanism of the phenomenon. Neither the presence of numerous unique tumor antigens nor the extraordinarily high frequency of selections can be explained on the basis of classic point mutations.

We therefore turned to a different means of deriving such variants using an agent—5-azacytidine—that is only weakly, if at all, mutagenic in eucaryotic cells but which can nevertheless drastically alter gene expression by causing DNA

| Tumor cells | Treatment | % 5-Methylcytosine | % of Control |
|-------------|-----------|--------------------|-------------|
| MDAY-D2     | No treatment | 3.90 ± 0.08 | |
| MDAY-D2     | 5 μM 5-azacytidine | 3.20 ± 0.15 | 82.0 |
| MDAY-D2     | 360 μg/ml EMS | 4.17 ± 0.15 | >100 |
| TA3         | No treatment | 3.15 ± 0.11 | |
| TA3         | 5 μM 5-azacytidine | 2.63 ± 0.05 | 84.0 |
| TA3         | 360 μg/ml EMS | 3.01 ± 0.02 | 96.2 |

* Cells were labeled with [6\textsuperscript{3}H]-uridine such that 2 \(\times 10^5\) cells/ml were incubated with 2 μCi/ml uridine for 18 to 24 h. The DNA was extracted and hydrolyzed as described by Flateau et al. (21) and outlined in Materials and Methods. Cells were labeled 4 d after treatment. Results are ± standard deviation from three separate determinations.
hypomethylation. The results presented herein describe our findings and suggest that the induction of tum" variants by treatment with conventional mutagens and 5-azacytidine most likely occurs by a common mechanism. Thus, we have demonstrated that: (a) treatment of tumor cell lines with 5-azacytidine can lead to the selection at high frequency of tum" variants; (b) those lines (e.g., TA3) in which >80% of the clones express the tum" phenotype after EMS treatment also gave rise to >80% tum" clones after 5-azacytidine treatment. Furthermore, if EMS was ineffective in deriving tum" clones from a particular tumor (e.g., MDAY-D2), the same was true after 5-azacytidine treatment; (c) 5-azacytidine-induced tum" variants grew readily in nude mice and induced strong CTL responses in culture—like the mutagen-induced tum" variants (7). We have also obtained recent evidence which suggests that the new 5-azacytidine-induced tumor antigens on the tum" clones are—as with EMS—highly polymorphic (Frost, unpublished observations). Despite these similarities, we found 5-azacytidine not to be significantly mutagenic when tested on our mouse tumor cell lines (Table III). This is in stark contrast to the results obtained with EMS.

In comparing the results using 5-azacytidine or the mutagens EMS or MNNG, it is important to note that Wilson and Jones (15) have demonstrated that these same mutagens can themselves cause hypomethylation of DNA. Similarly, Boehm and Drahovsky (16) have shown that MNNG can inhibit DNA methylation in viable human leukemia cells. These latter authors have also found hypomethylation of DNA in clones of P815 mastocytoma cells derived after treatment in vitro with the alkylating agent/carcinogen N-acetoxy-N-2-acetylamino fluorene, i.e., AAAF (17). Since 5-azacytidine is a very strong hypomethylating agent but such a poor mutagen in eucaryotic cells (22), the most plausible mechanism for the observed in vivo effects reported here is the influence both types of agents (i.e., 5-azacytidine or the alkylating agent mutagens) may have on DNA methylation levels and patterns, although this remains to be definitively established. In this regard our initial analyses of the 5-methylcytosine content of DNA are too preliminary to make any conclusions.

Assessment of overall levels of 5-methylcytosine in DNA may however be uninformative (23) especially since even small decreases may be sufficient to cause transcriptional activation of genes necessary for the expression of tum" phenotype. Other assays, and the examination of many independent clones (17) using restriction endonucleases and cDNA gene probes may be necessary to establish any clear relationship between DNA hypomethylation and the generation of tum" variants by mutagens. Thus, until gene probes for the appropriate tumor antigens exist, it will be impractical to evaluate this possibility. It should also be noted that even in a situation where carcinogen or mutagen treatment of tumor cells leads to an overall higher level of 5-methylcytosine content in the cells, many of the cells (i.e., clones) in the population may actually be hypomethylated (17). Thus, even the small (4%) decrease in methylcytosine content of EMS treated cells may be "significant" in the sense that it may be a reflection of far greater decreases in the many individual clones of the EMS-treated cells. It is also worth emphasizing that some authors have reported rapid hypomethylation followed by an equally rapid de novo methylation in some 5-azacytidine–induced tumor lines, e.g., Friend virus leukemia cells (24). In contrast, others report recovery
of methylation levels is far more protracted, e.g., 10–12 wk in AKR lymphoma cells (25). Just where our lines stand in this connection remains to be determined.

It is therefore clear that there are many complexities to be considered when evaluating the methylation status of the treated tumor cell populations. Nevertheless, we feel that the most plausible hypothesis, at this time, to account for our findings is the induction of DNA hypomethylation and subsequent transcriptional activation of dormant genes coding for tumor antigens. This hypothesis also has the attractive feature of providing an explanation for why many tum− clones revert to the tum+ phenotype, as described here and reported previously by us (7, 26). Such reversions, which are sometimes rapid (26), were difficult to explain on the basis of a mutational (i.e., base sequence change in DNA) origin for tum− clones. However, they are much more understandable in the context of what is known about the heritability of altered methylation patterns and levels, namely that they are somatically inherited with a high but not perfect fidelity (14, 25) so that they can rapidly, or more gradually, return to normal, or near normal, levels. Hence the term "stable" should be applied loosely to heritable phenotypic changes brought about by hypomethylation. Methylation levels may return to normal values over a period of days, weeks, or months, with a resultant reversion of certain induced phenotypic traits, as shown recently by Gasson et al. (25). These authors found glucocorticoid-sensitive clones derived from a 5-azacytidine–treated steroid-resistant mouse lymphoma line. Such glucocorticoid-sensitive clones were generated at very high frequency (10%), but gradually reverted to a resistant phenotype over a period of 2–3 months in culture (25). We have noted similar sorts of phenotypic reversions in our tum− variants, whether induced by 5-azacytidine or EMS (26).

We would also like to stress the implications these findings may have in regard to possible "epigenetic" origins of tumor progression (26–29). Gradual hypomethylation of DNA, possibly caused by carcinogen accumulation, chemotherapeutic drugs, or local environmental conditions, could result in progressive and random activation of many genes, thus potentially resulting in neoplastic cell populations of increasing diversity (26, 27, 29). From this population, the emergence of new (and possibly more malignant) subpopulations having heritable changes (of varying stability) in a particular phenotype, or set of phenotypes, could emerge by selection pressures or simple competitive advantages. In some cases, the change may render the cells less malignant because of, for example, an immunological alteration (as shown here), whereas in other cases, the genes activated may make the cells more malignant. Indeed, we have noted that among our 5-azacytidine–treated clones which remain tumorigenic, some are actually more malignant than the parent tumor, as defined by metastatic aggressiveness (26). L. Olsson2 has reported similar findings. Thus, it would appear that 5-azacytidine treatment of tumor cell populations can cause either a short-term or long-term heritable decrease in relative aggressiveness (as defined by progressive growth at the site of inoculation), or an increase, based on their ability to metastasize. These opposing changes can be detected by the testing of many

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1. Olsson, L., and J. Forchhammer. Induction of the metastatic phenotype in a mouse tumor model by 5-azacytidine and characterization of an antigen associated with metastatic activity. Submitted for publication.
individual clones derived from 5-azacytidine–treated parent tumor cell populations.

Summary

Highly immunogenic "tum-" (non-tumorigenic in normal syngeneic hosts) clonal variants can be selected from a variety of poorly immunogenic and highly tumorigenic mouse cell lines at very high frequencies (e.g., >80%) after treatment in vitro with chemical mutagens such as ethyl methanesulfonate (EMS) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). We herein demonstrate that the same result can be obtained with the poorly mutagenic cytidine analogue, 5-azacytidine, a strong DNA hypomethylating agent. 5-Azacytidine and EMS were equally and comparably effective, or ineffective, in inducing tum- variants from three different highly tumorigenic mouse cell lines. Like mutagen-induced tum- variants, those obtained after 5-azacytidine treatment generated usually strong cytolytic T lymphocyte (CTL) responses in vitro, and could grow in immunosuppressed (nude mouse) hosts. However, pretreatment of the tumor cell lines with 5-azacytidine did not cause significant increases in mutations at several independent drug-resistant gene loci, whereas EMS did. It is known that treatment of cells with 5-azacytidine can induce transcriptional activation of "silent" genes through a reduction of DNA 5-methylcytosine content, a process that can also be effected by mutagenic DNA alkylating agents such as EMS and MNNG. We therefore hypothesize that an "epigenetic" mechanism (DNA hypomethylation) leading to activation and expression of genes coding for potential tumor antigens is involved in the generation at high frequency of tum- variants after "mutagen" treatment. The implications of these findings to mechanisms of tumor progression and the generation of tumor heterogeneity are discussed.

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References

1. Boon, T., and O. Kellerman. 1977. Rejection by syngeneic mice of cell variants obtained by mutagenesis of a malignant teratocarcinoma line. Proc. Natl. Acad. Sci. USA. 74:272.
2. Van Pel, A., M. Georlette, and T. Boon. 1979. Tumor cell variants obtained by mutagenesis of a Lewis lung carcinoma cell line: immune rejection by syngeneic mice. Proc. Natl. Acad. Sci. USA. 76:5282.
3. Uyttenhove, C., J. Van Snick, and T. Boon. 1980. Immunogenic variants obtained by mutagenesis of mouse mastocytoma P815. I. Rejection by syngeneic mice. J. Exp. Med. 152:1175.
4. Boon, T., J. Van Snick, A. Van Pel, C. Uyttenhove, and M. Marchand. 1980. Immunogenic variants obtained by mutagenesis of mouse mastocytoma P815. II. T lymphocyte–mediated cytolysis. J. Exp. Med. 152:1184.
5. Van Pel, A., and T. Boon. 1982. Protection against a non-immunogenic mouse
leukemia by an immunogenic variant obtained by mutagenesis. *Proc. Natl. Acad. Sci. USA.* 79:4718.

6. Van Pel, A., F. Vessiere, and T. Boon. 1983. Protection against two spontaneous mouse leukemias conferred by immunogenic variants obtained by mutagenesis. *J. Exp. Med.* 157:1992.

7. Frost, P., R. S. Kerbel, E. Bauer, R. Tartamella-Biondo, and W. Cefalu. 1983. Mutagen treatment as a means for selecting immunogenic variants from otherwise poorly immunogenic malignant murine tumors. *Cancer Res.* 43:125.

8. Kerbel, R. S. 1979. Immunologic studies of membrane mutants of a highly metastatic murine tumor. *Am. J. Pathol.* 97:609.

9. Hewitt, H. B., E. R. Blake, and E. S. Walder. 1976. A critique of the evidence of active host defense against cancer based on personal studies of 27 murine tumors of spontaneous origin. *Br. J. Cancer.* 33:241.

10. Hewitt, H. B. 1982. Animal tumor models and their relevance to human tumor immunology. *J. Biol. Resp. Modifiers.* 1:107.

11. Kerbel, R. S., and P. Frost. 1982. Heritable alterations in tumor cell immunogenicity. *Immunology Today.* 3:34.

12. Razin, A., and A. D. Riggs. 1980. DNA methylation and gene function. *Science (Wash. DC).* 210:605.

13. Riggs, A. D., and P. A. Jones. 1983. 5-Methylcytosine, gene regulation, and cancer. *Adv. Cancer Res.* 40:1.

14. Wigler, M., D. Levy, and M. Peruchio. 1981. The somatic replication of DNA methylation. *Cell.* 24:33.

15. Wilson, V. L., and P. A. Jones. 1983. Inhibition of DNA methylation by chemical carcinogens in vitro. *Cell.* 82:239.

16. Boehm, T. L. J., and D. Drahovsky. 1983. Alteration of enzymatic methylation of DNA cytosines by chemical carcinogens: a mechanism involved in the initiation of carcinogenesis. *J. Natl. Cancer Inst.* 71:429.

17. Boehm, T. L. J., D. Grunberger, and D. Drahovsky. 1983. Aberrant de novo methylation of DNA after treatment of murine cells with N-acetoxy-N-2-acetylaminofluorene. *Cancer Res.* 43:6066.

18. Frost, P., R. S. Kerbel, and R. Tartamella-Biondo. 1981. Generation of highly metastatic tumors in DBA/2 mice: oncogenicity of A strain tumor cells. *Invasion & Metastasis.* 1:22.

19. Kerbel, R. S., R. R. Twiddy, and D. M. Robertson. 1978. Induction of a tumor with greatly increased metastatic growth potential by injection of cells from a low H-2 heterozygous tumor cell line into an H-2 incompatible parental strain. *Int. J. Cancer.* 22:583.

20. Gillin, G. D., D. J. Raufa, A. C. Beaudet, and G. T. Caskey. 1972. 8-Azaguanine resistance in mammalian cells. I. Hypoxanthine-guanine phosphoribosyl transferase. *Genetics.* 72:234.

21. Flateau, E., E. Bogenmann, and P. A. Jones. 1983. Variable 5-methylcytosine levels in human tumor cell lines and fresh pediatric tumor explants. *Cancer Res.* 43:4901.

22. Landolph, J. R., and P. A. Jones. 1982. Mutagenicity of 5-azacytidine and related nucleosides in C3H/10T1/2 clone 8 and V79 cells. *Cancer Res.* 42:817.

23. Doerfler, W. 1983. DNA methylation and gene activity. *Annu. Rev. Biochem.* 52:93.

24. Creusot, F., G. Acs, and J. K. Christman. 1983. Inhibition of DNA methyltransferase and induction of Friend Erythroleukemia cell differentiation by 5-azacytidine and 5-Aza-2'-deoxycytidine. *J. Biol. Chem.* 257:2041.

25. Gasson, J. C., J. Rhyden, and S. Bourgeois. 83. Role of de novo DNA methylation in the glucocorticoid resistance of a T-lymphoid cell line. *Nature (Lond.)*. 302:621.
26. Kerbel, R. S., P. Frost, R. Liteplo, D. Carlow, and B. E. Elliott. 1984. Possible epigenetic mechanisms of tumor progression: induction of high frequency heritable but phenotypically unstable changes in the tumorigenic and metastatic properties of tumor cell populations by 5-azacytidine treatment. *J. Cell Physiol.* In press.

27. Frost, P., and R. S. Kerbel. 1983. A possible epigenetic mechanism of tumor progression: the role of DNA hypomethylation. *Cancer Metastasis Rev.* 2:375.

28. Feinberg, A. P., and B. Vogelstein. 1983. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature (Lond.)* 301:89.

29. Gama-Sosa, M. A., V. A. Slagel, R. W. Trewyn, R. Xoenhandler, K. C. Kuo, C. W. Gehrke, and M. Ehrlich. 1983. The 5-methylcytosine content of DNA from human tumors. *Nucleic Acids Res.* 11:6883.