Effects of Mutagens on the Immunogenicity of Murine Tumor Cells: Immunological and Biochemical Evidence for Altered Cell Surface Antigens

Peter Altevogt,1 Paul von Hoegen, Sabine Leidig, and Volker Schirrmacher

Institute for Immunology and Genetics, German Cancer Research Center, D-6900 Heidelberg, Federal Republic of Germany

ABSTRACT

Eb lymphoma cells were subjected to treatment in vitro with the alkylating mutagen N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) and then cloned by limiting dilution. When tested in vivo for tumorigenicity in groups of syngeneic DBA/2 mice, 6 from 18 clones were found to be strongly reduced (turn- phenotype). The other clones showed only moderate or no change in tumorigenicity compared to the untreated control. All clones were able to grow in 400-rad-irradiated mice. Mice in which MNNG clones had regressed were able to generate tumor-specific cytolytic T-lymphocytes in vitro. Limiting dilution analysis indicated that 3 of 4 MNNG clones analyzed in detail displayed additional antigenic determinants that were detected by cytolytic T-lymphocytes. These data thus provided evidence for increased immunogenicity of some of the MNNG clones.

Membrane proteins of MNNG clones and original Eb cells were compared biochemically after metabolic labeling with [35S]methionine, TX114 solubilization, and electrophoretic separation. Two-dimensional gel maps revealed a general quantitative decrease in the expression of membrane proteins in MNNG clones. In addition, several proteins were only found in MNNG clones but not in untreated cells. Two membrane proteins of molecular weight 22,000 and 38,000 were greatly increased in expression in all MNNG clones but could be detected at a low level in the original Eb cells.

MNNG is known to be a strong mutagenic agent, but it can also interfere with DNA methylation and cause transcriptional activation of genes. We suggest that amplified cell surface structures may be the consequence of such transcriptional activation and could be involved in altered immunogenicity.

INTRODUCTION

Chemical carcinogens cause a wide range of damaging alterations to DNA. Alkylating mutagens such as MNNG,2 ethylmethanesulfonate, or ethylnitrosourea can chemically modify DNA, causing mutagenic effects by base conversion; these agents can also interfere with DNA methylation, thereby causing heritable changes in 5’-methylcytosine pattern (1). Since the pattern of 5’-methylcytosine residues in DNA is thought to be associated with gene expression in eukaryotic cells (2, 3), it has been suggested that changes in these patterns may be related to the oncogenic activities of some chemical carcinogens (4).

The effects exerted by mutagens on neoplastic cells were studied extensively by Boon (5). He showed that mutagen treatment in vitro of murine tumor cell lines can drastically alter their immunogenicity. When the surviving portion of MNNG-treated cells was cloned and the clones were subsequently injected into syngeneic recipient mice, quite often a high percentage of clones lost their ability to form progressively growing tumors (turn- phenotype (6)). The basis of this phenomenon could be shown to be an immunological one, since turn- clones readily formed tumors in immunosuppressed animals, like X-irradiated or nude mice (5, 6). Most importantly, it was noted that turn- cells could greatly enhance T-cell-mediated cytolysis in recipient mice, in comparison to non-mutagenized parental tumor cells. By detailed analysis in vitro with CTL clones, it was found that many turn- clones derived from a particular mutagenized parental line displayed new individual tumor-specific antigens (5). It was implied that these new tumor antigens on turn- clones, together with the already existing antigens on the original tumor, were the reason for the in vivo rejection of the variant cells (5).

Recently it was shown by Frost et al. (7) that 5’-azacytidine can also induce strongly immunogenic tumor cell variants. 5’-Azacytidine appears to be only weakly mutagenic or non-mutagenic (7, 8) and can cause hypomethylation of DNA which can lead to transcriptional activation of individual genes (3, 4) and sometimes even entire developmental programs (9, 10). It was suggested that the induction of turn- variants by treatment with alkylating mutagens and 5’-azacytidine is probably based on a common mechanism via DNA hypomethylation (7). According to this hypothesis (7) and as also implied by Boon’s work (5), strongly immunogenic variant cells should display new or altered cell surface components possibly involved in increased immunogenicity. Such cell surface structures have so far not been described in biochemical terms.

We have investigated cell surface-associated changes occurring in turn- clones that were established after MNNG treatment of mouse Eb lymphoma cells. Our results show that some of these clones have acquired new tumor antigens, as recognized by cytotoxic T-lymphocytes. Furthermore, we present biochemical evidence that mutagen treatment affects the expression of many cell surface antigens. Some surface components are strongly amplified, while others are reduced in expression.

MATERIALS AND METHODS

Animals and Tumor Cells. DBA/2 mice (6–8 weeks) were obtained from Bomholm (Denmark) or from the DKFZ animal breeding facilities. Eb is the Heidelberg subline of the L5178YE lymphoma. ESb is a spontaneous high metastatic variant of Eb. The etiology of ESb tumor cells has been described (11). Cells were maintained in tissue culture medium RPMI 1640 with penicillin (100 units/ml), streptomycin (100 µg/
Treatment of Cells with MNNG. For mutagen treatment the original uncloned batch of Eb 288 cells was used.

Treatment of cells with MNNG was performed as described by Boon and Kellermann (6). Briefly, 3 x 10^6 cells in 5 ml were incubated in culture medium in the presence of MNNG (3 μg/ml) for 1 h at 37°C. After this period, cells were washed twice and incubated for 24 h in fresh medium. Surviving cells were counted and cloned by limiting dilution with irradiated syngeneic spleen cells as filler cells.

Assessment of the tum- phenotype. Individual clones from Eb-MNNG-treated cells were injected subcutaneously at a dose of 2 x 10^5 cells into the backs of syngeneic normal or 400-rad-irradiated DBA/2 mice. After this pre-screening, the same dose of cells were given into groups of 8 DBA/2 mice. Tumor growth or regression was followed for 100 days. Growth rates of all Eb-MNNG-clones in vitro were slightly reduced in comparison to nontreated Eb cells irrespective of their tum- or tum+ phenotype in vivo. Growth rates in irradiated mice were comparable.

Analysis of T-cell Cytotoxicity. Animals in which individual Eb-MNNG clones had regressed were challenged again with 2 x 10^5 4000-rad- irradiated tumor cells of the same clone. After 7 days, spleen cells were removed and restimulated in vitro as described (12). Bulk culture generated cytotoxicity was measured in a standard 4-h ²¹Cr release assay on day 5 of restimulation (12).

Limiting Dilution Cultures. Limiting numbers of activated spleen cells (see above) were cultured together with 1 x 10^6 irradiated (2000 rad) syngeneic DBA/2 spleen cells and 3 x 10^6 mitomycin C-inactivated tumor cells (stimulator cells) in 96-well round-bottom microtiter plates. 24 replicates were plated per cell concentration. Each well received 0.2 ml culture medium (10% fetal bovine serum) and rat concanavalin A supernatant (vol/vol) was found to induce specific CTL proliferation. The plates were incubated in an isolated box under standard conditions (37°C, 5% CO₂, 95% humidified air) to keep culture conditions stable. After 8 days the cultures were split into 2 aliquots and analyzed in a standard ¹¹Cr release assay on tumor target cells. For the estimation of spontaneous release, each plate contained cultures which did not receive responder cells.

Statistical Analysis. The methods used for calculation of CTL precursors is described in detail elsewhere (13). Briefly, microcultures were scored as positive when ³¹Cr-release values exceeded the arithmetic mean of the counts of the low controls plus their 3-fold standard deviation. Minimal estimates of CTL-precursor frequencies were calculated by the statistical method of maximum likelihood (14). For both frequency estimates, the 95% confidence limits were calculated. The P-value of maximum likelihood goodness of fit to the single-hit Poisson model was determined. A P-value less than 0.05 indicates the data of the assay are not compatible with the single-hit Poisson model.

The global test for homogeneity of independent slopes (22) was used to determine whether or not resulting frequencies differ at the significant levels α = 0.05. With a P-value <0.05, the hypothesis of homogeneity has to be rejected.

Labeling of Cells and TX114 Extraction. Cells (5 x 10^7) in 200 μl methionine-free RPMI 1640 were labeled for 5 h with 100 μCi of [³⁵S]-methionine (SU204; Amersham, Braunschweig, Federal Republic of Germany). After labeling, cells were washed once in 1 ml Dulbecco’s phosphate-buffered saline, and the dry pellet was solubilized in ice for 30 min in 100 μl solubilization buffer containing 10 mm Tris/HCl, pH 7.4, 150 mm NaCl, and 1% TX114. The solubilization mixture was centrifuged for 15 min at 30,000 x g at 20°C. The clear supernatant was removed, and TX114-soluble proteins were prepared as described by Bordier (15). Briefly, the supernatant was layered on top of 500 μl of Tris/HCl, pH 7.4, containing 6% sucrose and 0.06% TX114 in Eppendorf tubes. The tubes were kept on ice for 5 min, giving rise to phase separation. Tubes were then centrifuged for 5 min at 1000 x g. The clear supernatant (100 μl) was removed from the top of the sucrose cushion; TX114 was added to a final concentration of 0.5%, and the tubes were kept on ice for 5 min. Samples were put again on the original sucrose cushion, and heating and centrifugation steps were repeated as described above. Finally, the TX114 phase was recovered from the bottom of the tube and diluted 5-fold with distilled water. Trichloroacetic acid-precipitable counts were determined, and the samples were prepared for 1- or 2-dimensional gel electrophoresis.

One- and 2-dimensional Gel Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 15% polyacrylamide gels using an acrylamide/bis-ratio of 20:1 and the Laemmli buffer system (16). Two-dimensional gel electrophoresis was carried out as described by O’Farrell (17) and as modified by Bravo et al. (18). Five x 10^5 trichloroacetic acid-precipitable cpm were applied to 140 x 1.7-mm glass tubes for the first dimension using a mixture of LBK ampholines 3.5–10 and 5–7 (final concentration, 2%; ratio, 0.33:1.66). After focusing, gels were extruded from the glass tubes and equilibrated in 10 ml of O’Farrell’s equilibration buffer for 15 min. Second-dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done as described above. Gels were fixed and processed for fluorography as described by Bonner and Laskey (19).

RESULTS

In Vivo Characterization of MNNG-treated Cells. Eb mouse lymphoma cells (Heidelberg subline of L5178YE) and the original L5178YE cells were subjected to a single treatment with the alkylating mutagen MNNG. After treatment, cells were cultivated for 24 h and then cloned by limiting dilution. Sixteen clones were established from Eb (subsequently called Eb-MNNG) and two clones from L5178YE cells. Growing clones were injected at a dose of 2 x 10^5 cells s.c. into the backs of syngeneic DBA/2 mice in order to assess their tumorigenicity. For control, we inoculated the untreated Eb parental cells and 2 clones thereof, the highly metastatic variant ESb and its tumor antigen negative subline 828. As can be seen from the results in Table 1, many of the MNNG-treated clones displayed greatly decreased tumorigenicity in comparison to control cells. Eb-MNNG clones 5, 6, 7, 8, and 9 either totally or to a very large extent lost their ability to produce tumors in syngeneic mice. These clones, as all others, were however able to grow as progressive tumors in 400-rad-X-irradiated mice. According to the terminology of Boon and Kellermann (6), we decided to designate the phenotype of Eb-MNNG clones 5, 6, 7, 8, and 9 and L5178YE-MNNG clone 2 as tum–. The phenotypic stability of these clones has been assessed by repeated injection into mice and has been found to be stable for over 5 months in culture.

Antigenicity of tum– Clones Derived after MNNG Treatment. In order to analyze whether the tum– clones had changed their antigenicity in comparison to non-treated cells, we analyzed the ability of regressor mice of particular clones to mount CTL responses. After appropriate secondary stimulation in vitro, splenic effector cells from some mice were tested for their ability to lyse target cells from either the respective clone used for induction or the parental Eb cells. As seen in Chart 1, some of the bulk culture-generated CTL populations lysed the respective clone more efficiently than did the untreated Eb parental cells.

Limiting Dilution Analysis of CTL Effector Cells. One reason
why some Eb-MNNG clones were more efficiently lysed than parental Eb cells could be that these Eb-MNNG clones express tumor antigens in addition to those being expressed by Eb cells (11). In order to test this assumption, spleen cells from regessor mice were tested by limiting dilution analysis. Varying numbers of splenic lymphocytes were seeded in microtiter plates and restimulated with the respective Eb-MNNG clone for 8 days. At the end of cultivation, wells were split and tested in an $^{51}$Cr-release assay for cytotoxic activity on the respective Eb-MNNG clone and on parental Eb cells. For this analysis, Eb-MNNG clones 1, 7, 8, and 9 were chosen according to the results of Chart 1. The data in Chart 2 show that under limiting dilution conditions of effector cells, two populations of CTL can be distinguished. One group lysed the Eb-MNNG clone and parental Eb target cells equally well, and a second group lysed only Eb-MNNG clones. These two populations of effector CTL could only be detected in spleen cells derived from regessor mice given injections of Eb-MNNG clones 7, 8, and 9. Thus, these animals had developed CTL-precursor populations that after in vitro stimulation developed CTL effectors detecting new tumor-antigens on Eb-MNNG-treated tumor cell clones. Eb-MNNG clones 7, 8, and 9 also displayed a tum- phenotype, as seen in Table 1. In contrast, all p-CTLs induced by Eb-MNNG clone 1 lysed clone 1 and parental Eb cells, but no extra specificities directed against Eb-MNNG clone 1 could be detected by limiting dilution analysis. A statistical evaluation of the results generated in limiting dilution assays is given in Table 2. This table shows that CTL precursors against MNNG tumor cells occurred with quite similar frequencies of 1/12,000 to 1/15,000. Precursor frequencies against the Eb-tumor-associated transplantation antigen common to all clones analyzed were from 1/28,000-1/36,000 for all Eb-MNNG clones analyzed, and they do not differ significantly according to the global test for homogeneity of independent slopes.

### Fine Specificity Analysis of CTLs Induced by Eb-MNNG Clone 8 Tested on other Eb-MNNG Clones

Studies by Boon (5) have indicated that newly induced tumor antigens on mutagen-treated clones are polymorphic. We have studied a possible polymorphism by analyzing the fine specificity of Eb-MNNG clone 8-induced CTL in limiting dilution. DBA/2 mice were sensitized against the tumor clone by injection of viable clone 8 cells into the ear as described previously (12). Splenic lymphocytes were restimulated in vitro with Eb-MNNG clone 8 cells under limiting dilution conditions. The wells of limiting dilution cultures were split into replica on day 6 and tested for cytotoxicity on 4 different targets (Eb, Eb-MNNG clone 8, clone 7, clone 9). 144 wells were analyzed, of which 40% lysed significantly only Eb-MNNG clone 8 but not parental Eb cells. 80% of these clone 8-specific wells also lysed Eb-MNNG clone 7 targets, but only 43% were cytolytic to Eb-MNNG clone 9 targets. These data suggest that target structures on clones 8 and 7 are more closely related than are those expressed by Eb-MNNG clone 9. A more detailed analysis of CTL determinants is currently being performed on the clonal level.³

### Biochemical Analysis of Membrane Proteins by 2-dimensional Gel Electrophoresis

The discovery of additional antigenic determinants on Eb-MNNG clones by CTL analysis prompted us to look for cell surface changes by biochemical techniques. For this, Eb parental tumor cells and Eb-MNNG clones 1 and 8 were labeled with $^{35}$S)methionine and then solubilized in TX114. After phase condensation, the TX114 phase soluble proteins were isolated. This procedure has been found to enrich for hydrophobic proteins, most of which are membrane proteins (15). These proteins were analyzed by 2-dimensional gel analysis. Fig. 1 (A-C) displays 2-dimensional gel maps, and schematic drawings of the spots observed are given in Fig. 1 (D-F). By comparison of these maps, the following observations were made: (a) the 2-dimensional gel patterns from

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³ P. V. Hoegen, unpublished results.

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### Table 1

Assessment of tumorigenicity of MNNG clones and control cells in syngeneic recipient DBA/2 mice

<table>
<thead>
<tr>
<th>Tumor takes (%)</th>
<th>Positive tumors/total</th>
<th>Av. survival time</th>
<th>Growth in irradiated mice</th>
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<tr>
<td>EB: 288 TATA++</td>
<td>100/8/8</td>
<td>21/14-27</td>
<td>+</td>
</tr>
<tr>
<td>EB 828 TATA++</td>
<td>100/8/8</td>
<td>15/14-18</td>
<td>+</td>
</tr>
<tr>
<td>EB 288 parental</td>
<td>100/8/8</td>
<td>32/24-46</td>
<td>+</td>
</tr>
<tr>
<td>EB clone 2941</td>
<td>88/7/8</td>
<td>56/38-62</td>
<td>+</td>
</tr>
<tr>
<td>EB clone 2940</td>
<td>88/7/8</td>
<td>57/39-63</td>
<td>+</td>
</tr>
<tr>
<td>EB-MNNG clone 1</td>
<td>57/4/7</td>
<td>34/22-47</td>
<td>+</td>
</tr>
<tr>
<td>EB-MNNG clone 2</td>
<td>63/5/8</td>
<td>44/37-50</td>
<td>+</td>
</tr>
<tr>
<td>EB-MNNG clone 3</td>
<td>63/5/8</td>
<td>40/32-50</td>
<td>+</td>
</tr>
<tr>
<td>EB-MNNG clone 4</td>
<td>29/2/7</td>
<td>47/25-69</td>
<td>+</td>
</tr>
<tr>
<td>EB-MNNG clone 5</td>
<td>14/1/7</td>
<td>56/37-56</td>
<td>+</td>
</tr>
<tr>
<td>EB-MNNG clone 6</td>
<td>0/0/8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EB-MNNG clone 7</td>
<td>0/0/9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EB-MNNG clone 8</td>
<td>25/2/8</td>
<td>31/21-41</td>
<td>+</td>
</tr>
<tr>
<td>EB-MNNG clone 9</td>
<td>13/1/8</td>
<td>37/34-49</td>
<td>+</td>
</tr>
<tr>
<td>EB-MNNG clone 10</td>
<td>50/4/8</td>
<td>34/35-49</td>
<td>+</td>
</tr>
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<td>EB-MNNG clone 11</td>
<td>44/4/9</td>
<td>48/28-92</td>
<td>+</td>
</tr>
<tr>
<td>EB-MNNG clone 12</td>
<td>63/5/8</td>
<td>68/45-52</td>
<td>+</td>
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<td>EB-MNNG clone 13</td>
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<td>67/45-68</td>
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<td>68/40-98</td>
<td>+</td>
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<td>EB-MNNG clone 15</td>
<td>75/6/8</td>
<td>65/33-93</td>
<td>+</td>
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<td>EB-MNNG clone 16</td>
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<td>44/35-53</td>
<td>+</td>
</tr>
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<td>+</td>
</tr>
<tr>
<td>LS178YE-MNNG clone 2</td>
<td>13/1/8</td>
<td>31/37-41</td>
<td>+</td>
</tr>
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</table>

* TATA, tumor-associated transplantation antigen.

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### Chart 1

Cytotoxic T-cell responses generated by Eb-MNNG clones. Animals that had regressed individual Eb-MNNG clones were challenged with $2 \times 10^{6}$ 5000-rad-irradiated identical clones. Spleens were removed after 7 days, restimulated in vitro with irradiated clones, and tested on day 5 of culture either on untreated parental tumor cells (C) or on the clone used for priming (A). Standard deviation for mean values given were <5%.
Chart 2. Analysis of p-CTL effector populations generated in syngeneic mice against selected Eb-MNNG clones in limiting dilution cultures. Lower panel, cytolytic activity of CTL clones is shown on the appropriate Eb-MNNG clone and on the parental Eb line. Dots on the diagonal indicate that both targets are lysed equally well. Upper panel, frequency estimation of p-CTL resulting from experiments shown above. The effector cells were tested in a split experiment on the Eb-MNNG clone used for stimulation and on the parental line Eb (Δ). A P-value >0.05 indicates that the data are consistent with the single-hit Poisson model.

Table 2
Frequency estimation of tumor-specific p-CTL in Eb-MNNG regressor mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Tumor cell</th>
<th>CTL-p frequency (1/n (ML)^a)</th>
<th>95% confidence limits (1/n)</th>
<th>P-value</th>
<th>P-value of frequency b</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBA/2a Eb 288 MNNG CI 1</td>
<td>Eb 288 MNNG CI 1</td>
<td>36,500</td>
<td>28,599</td>
<td>50,435</td>
<td>0.73</td>
</tr>
<tr>
<td>DBA/2a Eb 288 MNNG CI 7</td>
<td>Eb 288 MNNG CI 7</td>
<td>12,181</td>
<td>9,514</td>
<td>16,296</td>
<td>0.96</td>
</tr>
<tr>
<td>DBA/2a Eb 288 MNNG CI 8</td>
<td>Eb 288 MNNG CI 8</td>
<td>15,523</td>
<td>12,276</td>
<td>21,106</td>
<td>0.86</td>
</tr>
<tr>
<td>DBA/2a Eb 288 MNNG CI 9</td>
<td>Eb 288 MNNG CI 9</td>
<td>12,525</td>
<td>8,467</td>
<td>23,983</td>
<td>0.97</td>
</tr>
</tbody>
</table>

a ML, maximum likelihood.

b Each p-CTL determination is based on 7 cell concentrations and 24 replica wells per cell concentration tested. The frequency of p-CTL was determined by maximum likelihood.

c The global test for homogeneity of independent slopes was used to compare the frequency values on the parental line and the Eb-MNNG clone. P < 0.05 indicates that the frequency values of p-CTL tested on the two targets differ at the significant level α = 0.05.

d P > 0.05, indicating that the data are consistent with the single-hit Poisson model (see "Materials and Methods").

Eb cells revealed approximately 800 spots; those of Eb-MNNG clones 8 and clone 1, respectively, revealed 610 spots, as estimated by visual inspection. This decrease in synthesis or total disappearance of protein spots is most strikingly exemplified by comparing spots 1–7 (Fig. 1, D–F). Thus, overall, a decrease in complexity of membrane proteins between Eb- and MNNG-treated clones was noted; (b) we noted several proteins in MNNG-treated cells that were not detected in parental cells and other protein spots that were increased in expression. This is exemplified by spots 8–13. Most strikingly, Eb-MNNG-treated clones displayed an increased labeling of two proteins of molecular weight approximately 38,000 and 22,000 (subsequently referred to as p38 and p22) which were detectable in Eb parental cells at much lower rate of synthesis; and (c) comparison of MNNG clones with each other revealed only a limited degree of heterogeneity in some spots (data not shown). No major differ-
ences were noted between Eb-MNNG clones 1 and 8 shown here.

**Amplified Expression of p38 and p22 Proteins.** We focused our interest on p22 and p38 proteins. Both proteins appeared to be sensitive to proteolytic enzymes, since protease K treatment of radiolabeled intact cells abolished the appearance of these structures in TX114 extracts almost completely (data not shown). These results indicated that p22 and p38 are probably components of the outer cellular membrane. More Eb-MNNG clones were tested to see if this amplification in expression was a general phenomenon. Eb parental cells and clones of Eb cells were included in the analysis. Fig. 2A shows that all 10 analyzed Eb-MNNG clones showed increased labeling of p38 and p22. In contrast, clones derived from the parental line Eb did not display amplified expression of p38 and p22, indicating that the changes seen were not due to a clonal selection by the mutagen of high expressor clones but rather a consequence of mutagen treatment. The degree of amplification was estimated to be approximately 2-3-fold for p22 and from 3-5-fold for p38.

**DISCUSSION**

Studies on the immunogenicity of tumor cells and on the alteration of host antitumor immune responses is an area of intensive research. Two approaches have been put forward: (a) therapeutic manipulation of the immune system by immune response modifiers, elimination of suppressor cells or adoptive immunotherapy (20); and (b) changing the immunogenicity by “xenogenization” with viruses (21, 22), haptenization (23), or mutagen treatment (6). The latter approach has received increasing attention because of the demonstration that non-immunogenic mouse tumors can be rendered immunogenic (24, 25) and that mutagen-treated tumor cells with increased immunogenicity can confer protection to the original non-mutagenized tumor cell population (24-27). In this respect, mutagen treatment of tumor cells in vitro is likely to gain importance in immunotherapeutic approaches against neoplastic cells.

Our main interest in this study has been to investigate changes which may be detectable at the tumor cell surface after mutagen treatment, especially since there exists as yet little information about this. This approach appears to be of obvious importance, since any change in immunogenicity should be linked to the cell surface. The results presented in the first part of our work show evidence for the existence of new antigenic determinants on mutagen-treated Eb tumor cell clones, which are detectable by CTL analysis. These results are well in line with findings in other tumor systems using MNNG and different mutagens, as well as other assay systems (5, 6, 28-32). By limiting dilution analysis, we found these new antigenic determinants on some but not all Eb-MNNG clones analyzed. The new determinants detected on clones 7, 8, and 9 appeared to be dissimilar.

A biochemical comparison of membrane proteins from MNNG-treated clones and those from non-treated Eb parental cells revealed important differences. There was a substantial decrease in the overall number of detectable 2-dimensional gel spots for MNNG clones in relation to non-treated cells. Furthermore, we have detected several proteins on the surface of MNNG-treated cells that were not detected in the parental cells. Some proteins, like p38 and p22 proteins, were increased in expression and could be detected at lower levels also in non-treated Eb tumor cells. The amplification of p38 and p22 was noted to be a common feature of all MNNG clones analyzed.

We do not think that we can, as yet, correlate any of the additional 2-dimensional gel spots to the new immunogenic determinants on MNNG clones detected by CTL analysis. Neither comparison of 2-dimensional gel maps between Eb-MNNG clone 1 and 8 nor that between clone 7 and 1 (data not shown) revealed a component as a candidate. Several explanations could be given: (a) the most trivial one is that the gel conditions used are not capable of analyzing all membrane proteins of importance. For Hela cell total extracts, for example, it has been estimated that only approximately 50-60% of applied radioactivity can be resolved under these conditions; 4 and (b) new CTL determinants can often be induced by quite minute changes in the primary structure of a protein. This has most elegantly been demonstrated by the studies on the H-2^{a/b} mutants, where one or two amino acid exchanges on the H-2K molecule can give rise to new CTL recognized determinants (33). With regard to our analysis, this could mean that we simply missed these changes due to limited sensitivity.

The changes that were detected in MNNG cells could well, however, have influenced the immunogenicity of the tumor cells. Most of the clones established after MNNG treatment showed a decreased tumorigenicity in comparison to control cells. With respect to Eb-MNNG clone 1, at least, this decrease in tumoriogenicity could not be attributed to new tumor antigens detectable by CTL analysis. It is possible that some of the amplified cell surface components on mutagen-treated cells which were also found in clone 1 act as helper determinants and strengthen the immunological response of the host against already existing tumor antigens (34). The p38 and p22 or other proteins that are increased in expression on all MNNG clones in comparison to parental cells could be candidates for molecules carrying such helper determinants.

Little is known so far about the mechanisms by which alkylating mutagens may exert such a strong effect on the immunogenicity of tumor cells. O^6-Methylation of guanidine appears to account for most of the MNNG-induced mutagenic effects (1, 35). Following mutagen treatment, O^6-methylguanidine premutagenic lesions are induced in the DNA in large numbers. O^6-Methylguanidine can mispair with thymidine. Mispairing results in transitions from G-C to A-T base pairs in the next round of replication. In yeast, clusters of mutations within regions of DNA actively replicating have been found and have been used to study neighboring genes (36). Mutagenic effects on mammalian cells are highest in the S-phase of the cell cycle (37, 38). There are cellular repair mechanisms known that are antagonistic to the mutagenic effect (1). Furthermore, several reports have shown that DNA damage by alkylating mutagens can induce gene activation (39-42). It is possible that this activation involves interference with the enzyme cytosine-5-methyltransferase that has been found to be inhibitable by alkylating mutagens (43-46).

It is presently unknown what the relative contribution of these main mutagen effects is for the induction of the tum—phenotype. Frost et al. (7) have proposed that the high frequency of induction of this phenotype after mutagen treatment cannot be explained by single point mutations. In support of this notion, it was shown that 5'-azacytidine, a drug that interferes with cytidine methyl-
tion and is itself not mutagenic or only weakly mutagenic (7, 8), can induce the tum—phenotype at a similar high frequency as alkylating mutagens. These findings led to the proposal that the tum—phenotype may be induced in amplified protein expression, since we noted that very similar cell surface changes as seen in MNNG clones are induced in EB cells by treatment with 5'-azacytidine. EB-5-azacytidine-treated cell clones were also decreased in tumorigenicity. 6 It is tempting to speculate that genes for the amplified proteins should be candidates for such a proposed derepression mechanism and could possibly play a role in altered immunogenicity of tum—EB-MNNG clones.

Further characterization of cell surface proteins of tum—cells, studies on the transcriptional control of genes encoding these proteins and of their role in immunogenicity, should allow for the first time the investigation of the requirements for the tum—phenotype at the molecular level.

ACKNOWLEDGMENTS

We would like to thank Anja Kaun for competent help in the preparation of this manuscript.

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Fig. 1. Two-dimensional gel electrophoresis of [³⁵S]methionine labeled TX114-soluble membrane proteins from parental nontreated Eb cells and Eb-MNNG clones. Upper panel: A, Eb (untreated); B, Eb-MNNG clone 1; C, Eb-MNNG clone 8. Induced or amplified spots of MNNG clones are indicated by arrows. Lower panel: D–F, schematic drawings of A–C. Protein spots disappearing from the Eb map are indicated by boxes.
Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of p38 and p22 expression on MNNG clones and parental cells. Eb-MNNG clones 1–10, Eb clones 2940, 2941 and 2942, and parental Eb cells were labeled with [35S]methionine for 5 h. Triton TX114-soluble membrane proteins were prepared and analyzed on a 15% sodium dodecyl sulfate-polyacrylamide gel under reducing conditions. p38 and p22 proteins are indicated.
Effects of Mutagens on the Immunogenicity of Murine Tumor Cells: Immunological and Biochemical Evidence for Altered Cell Surface Antigens

Peter Altevogt, Paul von Hoegen, Sabine Leidig, et al.

*Cancer Res* 1985;45:4270-4277.

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