Heterogeneity and Clonal Variation Related to Cell Surface Expression of a Mouse Lung Tumor-associated Antigen Quantified Using Flow Cytometry

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ABSTRACT

Previous reports have established that line 1, a spontaneous BALB/c lung carcinoma, expresses a M, 180,000 tumor-associated surface antigen (TSP-180). In this study, using a monoclonal antibody and flow cytometry to quantify cell surface TSP-180 expression, we found that essentially all cells in a tissue culture-adapted line 1 population express TSP-180, but that the amount of TSP-180 expressed by cells is quite heterogeneous. Variation in amount of TSP-180 was found to be in part related to cell size heterogeneity, and to the expression of TSP-180 being cell cycle-dependent. The amount of surface-expressed TSP-180 correlated somewhat with cell size, and was greater on the average for cells in G2 cell cycle compartment. However, cells of a defined size and specific cell cycle stage still showed marked heterogeneity of expression. Even though the average amount of TSP-180 expressed per cell decreased during in vitro propagation, little change in heterogeneity was observed. To explore whether any TSP-180-related heterogeneity resulted from heritable variation of expression, 263 limiting dilution-derived line 1 clones were analyzed. The majority displayed, shortly after cloning, heterogeneous TSP-180 profiles and mean TSP-180 levels similar to those observed for the parent tumor. Occasionally, however, clones were isolated that again appeared as heterogeneous as the parent, but differed by as much as 3-fold in mean TSP-180 expression. Extensive passage did not substantially increase the low probability of isolating clones which differed in expression of TSP-180. Differences in TSP-180 expression among clones were found to be relatively stable upon passage, typically maintained after recloning, and large enough to influence clonal susceptibility to TSP-180-directed antibody and complement-mediated lysis. Heritable variation in TSP-180 expression among some clones was also shown to be independent of differences related to cell size, cell cycle, or expression of another line 1 surface antigen (P-100). We concluded that although clones demonstrating large heritable differences in TSP-180 expression can occasionally be isolated, line 1 TSP-180 heterogeneity is predominantly nonheritable, being similar to that present in recently cloned lines, quite stable during in vitro passage, and not totally accounted for by cell cycle or cell size variation.

INTRODUCTION

Tumor cell populations have frequently been observed to be heterogeneous with respect to a wide variety of phenotypic traits, e.g., metastatic potential (10, 19, 22), drug sensitivity (25, 26), karyotype (9, 25), and antigen expression (4, 5, 7, 9), an important consideration when evaluating tumor progression. Although those tumors which express TAAg can be antigenically distinguished from normal tissue, not all that display TAAg generate productive immune system responses. Heterogeneity in quantitative TAAg expression is of concern because immune system-mediated lysis of a tumor cell is typically related to the amount of antigen the cell expresses (17). Indeed, tumor development has been associated in some cases with the loss of TAAg (2, 27), and clearly, an immunogenic tumor could also survive in vivo if cells which express TAAg below a level required for effective immune system recognition are maintained.

This report will concentrate on the cell surface expression of a M, 180,000 glycoprotein (TSP-180) within line 1, a mouse lung carcinoma. TSP-180 was quantified using indirect immunofluorescence, using a previously described monoclonal antibody (12) and flow cytometry. TSP-180 has been identified on a limited number of murine lung carcinomas besides line 1, but has not been detected in normal tissue or tumors not of murine lung origin (13). We describe in this report various aspects related to the heterogeneous nature of TSP-180 expression evident within line 1. Since the selection of variant clones is believed to underly tumor progression (20), we were particularly interested in differentiating heritable from nonheritable TAAg heterogeneity.

MATERIALS AND METHODS

Tumor Cells. Line 1 is a murine type II alveolar cell carcinoma that arose spontaneously in a BALB/c female approximately 10 years ago (29). Experiments presented below were performed using an in vitro-maintained cell line developed from an animal tumor supplied by Dr. J. H. Yuhas. Proliferating cells from line 1 tumor mince were passed twice in culture before cryopreserved stocks were established. The line has a doubling time near 13 hr, and, like the in vivo-maintained tumor, is metastatic, weakly immunogenic, and highly malignant.

Tissue Culture. Line 1 and all clones were maintained in exponential growth using Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37° in a fully humidified atmosphere containing 10% CO2. Cell lines were split approximately 100:1 every 4 days, and periodically screened and demonstrated negative for Mycoplasma using Hoechst 33258 stain (6).

Cloning. Placing 0.2 ml of medium, which contained 1.5 cells/ml, in each well of a 96-well flat-bottomed tissue culture plate typically generated 20 to 30 positive wells/plate, and these were scored microscopically for single colony growth approximately 1 week after cloning. Cells from

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1 This work was supported by NIH Grant CA 28322.
2 Supported by NIH Grant 5 T32 GM07356. To whom requests for reprints should be addressed.
3 The abbreviations used are: TAAg, tumor-associated antigen; TSP-180, M, 180,000 murine lung tumor-restricted surface protein; P-100, M, 100,000 cell surface protein; FITC, fluorescein isothiocyanate; PI, propidium iodide; CV, coefficient of variation; PBS, phosphate-buffered saline (7.65 g NaCl, 1.27 g Na2HPO4, 0.1 g NaH2PO4, H2O, and 0.21 g K2HPO4 per liter of H2O); FALS, forward-angle light scatter; TF, time of flight.
single-colony-containing wells were then transferred to 10-mm tissue culture dishes, grown to near confluence, and replated prior to subsequent analysis. Cell lines referred to as cloned lines were cloned at least twice and were cryopreserved approximately 2 weeks after the second cloning.

**Monoclonal Antibodies.** The production, physical characterization, and binding specificity of the IgG2A rat monoclonal antibodies 133-13, 133-14, and 133-15 have been described previously (12). Briefly, antibody 133-13 recognizes a M, 180,000 glycoprotein (TSP-180), the expression of which appears limited to murine lung carcinomas. Antibody 133-14 binds to a M, 100,000 glycoprotein (P-100) that is expressed by normal and transformed cells. Antibody 133-15 was used as a negative staining control.

**Immunofluorescent Staining.** Surface antigens were labeled with FITC in V-bottomed microtiter plates held at 4° using a 2-step procedure. Exponential growth phase cells were harvested from culture using either 0.1% trypsin or, whenever trypsin-sensitive P-100 was labeled, cold citrate saline (134 m KCl, 0.15 mM Na citrate). Cells were then washed with citrate-saline containing 0.1% sodium azide and 2% bovine serum albumin (used for all wash steps and antibody dilutions), distributed at 2 million cells/well, and then incubated with 50 μl of primary antibody for 20 min, using twice the minimum concentration determined to be necessary for saturation (1:200 dilution of ascites). Cells were then washed twice, incubated for an additional 20 min with 50 μl of secondary goat anti-rat FITC-labeled F(ab')2 fragment (Cappel Laboratories, Cochranville, PA) under saturating conditions, washed, and analyzed within 1 hr.

**Quantitative Immunofluorescence and Flow Cytometry.** At least 10,000 cells/sample were individually analyzed for quantitative fluorescence using an EPICS V cell sorter (Coulter Electronics, Hialeah FL). PI was added (final concentration, 5 μg/ml) so that nonviable cells could be excluded from analysis (14). Only linear fluorescence data were collected. Mean fluorescence channels and CVs were determined from disc-stored histograms using computer programs developed initially by Salzman et al. (23) and extensively modified by Dr. J. F. Leary and Roy Robinson (University of Rochester). Average fluorescence values are expressed relative to a fluorescence standard, and were calculated using:

\[ F_s = F_0 \times \frac{50s}{50n} \times \frac{d_0}{g_0} \]

where \( F_s \) is the standardized mean fluorescence value of the sample; \( F_0 \), the uncorrected mean fluorescence channel; \( 50s \), an observed mean fluorescence channel of 50th bright fluorospheres arbitrarily chosen as the fluorescence standard; \( 50n \), the observed mean fluorescence of 50th bright spheres recorded when \( F_0 \) was measured; and \( d_0/g_0 \), the ratio of linear amplifier gain settings used for 50n and \( F_0 \) determinations. Since both the primary and secondary antibodies used during labeling were applied under saturating conditions, fluorescence intensity is linearly related to the amount of antigen expressed by a cell. A number proportional to the average amount of surface antigen expressed per cell can be obtained from the mean fluorescence value of a sample by subtracting the mean control fluorescence value. To calculate antigen density, the control-corrected fluorescence value of a sample was multiplied by 1000, and then divided by the average surface area per cell, calculated from mean Coulter volume, making the assumption cells are spherically shaped (area = 4.84 (volume)^0.5).

**Cell Size-correlated Data.** Surface-expressed TSP-180 and either FALS or TF were simultaneously evaluated using flow cytometry. TF represents the time required to traverse the laser beam, or "time of flight" (24). Measurements of TF were made inputting a pulse FALS signal to a module developed by Dr. J. F. Leary (15). Size calibration before use with 10.0- and 19.52-μm-diameter spheres ensured the TF output was directly proportional to particle diameter. Correlated TF values were obtained by reprocessing TF list mode data using the above-mentioned programs, which were also used in calculating bivariate display correlation coefficients.

**Cell Cycle Analysis.** Cells FITC-stained for TSP-180 were washed with PBS and resuspended in 1 ml of cold 70% ethanol. After fixation, 0.1 ml of a 50-μg/ml PI solution, 0.1 ml of a 1-mg/ml RNase solution, and 1 ml of water were added, and the mixture was held at 37° for 30 min. Prior to flow-cytometric analysis, the cells were resuspended in PBS. Correlated measurements of DNA-bound PI, FITC-labeled TSP-180, and cell size were obtained using a single 488-nm excitation source with the cytometer adjusted so that cells stained only with PI and cells stained only with FITC gave orthogonal signals on a red-green scatter plot. The percentage of cells in different cell cycle phases was determined from PI fluorescence data using a computer program called SFIT (8), which was provided by Dr. Phil Dean and modified at Rochester by R. H. Wilson.

**Coulter Volume.** Shortly after harvest, cells were passed through a Model ZBI Coulter Counter, which was connected to a Coulter Channelizer interfaced to an Apple II computer (Accucomp, Coulter Electronics). Microspheres (19.35-μm diameter) were used as volume standards. Volume histograms were stored on floppy discs and later analyzed for mean Coulter volume using the C-1000 channelizer program (Coulter Electronics).

**Complement-mediated Lysis.** Briefly, 2 million cells were incubated with 100 μl of a given dilution of 133-15 or 133-13 ascites for 20 min at 4°, washed, then incubated with 100 μl of a 1:15 dilution of rabbit anti-rat IgG (Cappel Laboratories, Cochranville, PA) for 20 min, washed, resuspended in 1 ml of a 1:15 dilution of rabbit complement made in medium, and held for 30 min at 37°. Cold PBS (1 ml) was then added along with 0.2 ml of a PI solution (final concentration, 5 μg/ml). The number of stained nonviable cells in 10,000 was determined using the flow cytometer set to record PI-associated red fluorescence and FALS.

**RESULTS**

**Heterogeneity of TSP-180 Expression.** Previous work established that line 1 expresses TSP-180, but did not indicate how TSP-180 expression varied throughout the cell population. Labeling surface expressed TSP-180 with FITC followed by flow-cytometric analysis reveals that essentially all line 1 cell lines fluoresce above the negative control and thus display TSP-180 (Chart 1). However, since cell fluorescence is linearly related to the amount of TSP-180 a cell has on its surface, the wide fluorescence histogram associated with the parent population implies that considerable heterogeneity of expression is present.

To show that this range of expression is not caused by orientation variation or deformation of cells during flow, cells with a restricted TSP-180 distribution were analyzed concurrently with the parent. The TSP-180-restricted cells were selected from
the line 1 parent population immediately before it was evaluated for TSP-180 using the sorting feature of our flow cytometer set to deflect cells which displayed fluorescence values within a 10-channel window (Channels 65 to 75). The fluorescence distribution associated with this sorted population has a CV of 16 compared to the parent populations value of 39, and as shown displays much less overall TSP-180 heterogeneity (Chart 1). This clearly demonstrates that line 1 TSP-180 heterogeneity is substantially greater than any antigenic variation which may be generated artificially by flow cytometry.

Heritable and Nonheritable Antigenic Variation. Clones isolated by limiting dilution from line 1 can exhibit marked differences in cell surface expression of TSP-180 (Table 1; Chart 2), indicating the same line 1 TSP-180 heterogeneity is heritable. The maximal clonal difference in mean TSP-180 expression that we observed among 263 clones was approximately 3-fold, and all clones expressed a non-zero level of TSP-180. Further information concerning the presence or absence of TSP-180-negative variants was derived from a cloning device used in conjunction with the flow cytometer (Autoclone; Coulter Electronics) set to sort single TSP-180 stained cells, displaying control fluorescence values, into individual cloning wells. All 16 clones grown up from these sorted cells were found to be TSP-180-positive shortly after cloning (not shown). Heritable TSP-180 variation within line 1 appears, therefore, to be restricted to quantitative rather than qualitative differences in antigen expression.

Considerable TSP-180 heterogeneity exists even within cloned line 1 populations as soon as 2 weeks after cloning (Chart 2). Because the clonal TSP-180 histograms all have CVs within 2% of the CV associated with the TSP-180 distribution of the line 1 parent, flow-cytometric analysis implies that the heterogeneity of TSP-180 expression within line 1 is similar to that observed in a clonally sorted population (variations in histogram width apparent in Chart 2 are simply the result of differences in mean levels of expression among clones, and as such do not reflect true differences in antigenic heterogeneity). Because cloned cell lines represent populations with minimal heritable heterogeneity, finding similar levels of TSP-180-related heterogeneity in the parent and clones suggests that most line 1 TSP-180-related heterogeneity is nonheritable. The predominance of nonheritable TSP-180 variation in line 1 is also indicated by our finding the vast majority of 263 clones evaluated for TSP-180 expression essentially indistinguishable from the parent (see also Table 3).

Determinants of Clonal TSP-180 Variation. Differences in TSP-180 expression among some clones can partially be explained by cell size; e.g., clones A11, C10, and C6 have TSP-180 density values that are similar (Table 1) indicating that cell size and TSP-180 are sometimes correlated. Indeed, most of the cloned lines we isolated with larger cells had TSP-180 levels.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Coulter volume (fl)</th>
<th>TSP-180</th>
<th>P-100</th>
<th>TSP-180 densitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>A11</td>
<td>2000 ± 100b</td>
<td>107 ± 3</td>
<td>440 ± 45</td>
<td>140</td>
</tr>
<tr>
<td>C10</td>
<td>1800 ± 300</td>
<td>93 ± 8</td>
<td>470 ± 35</td>
<td>130</td>
</tr>
<tr>
<td>A5</td>
<td>1900 ± 100</td>
<td>55 ± 7</td>
<td>480 ± 15</td>
<td>85</td>
</tr>
<tr>
<td>C6</td>
<td>3600 ± 200</td>
<td>177 ± 10</td>
<td>860 ± 70</td>
<td>150</td>
</tr>
<tr>
<td>C25</td>
<td>3800 ± 300</td>
<td>88 ± 8</td>
<td>730 ± 30</td>
<td>74</td>
</tr>
<tr>
<td>A13</td>
<td>3700 ± 200</td>
<td>61 ± 8</td>
<td>930 ± 70</td>
<td>53</td>
</tr>
<tr>
<td>Line 1</td>
<td>1800 ± 200</td>
<td>70 ± 7</td>
<td>480 ± 60</td>
<td>98</td>
</tr>
</tbody>
</table>

a Average ± S.E. of 3 control-corrected mean fluorescence values standardized to 50th bright microspheres, mean fluorescence Channel 164. The 3 measurements were made 2 days apart using different samples. Mean negative control fluorescence was approximately 10 for the smaller clones and 17 for the larger-sized clones.

b Arbitrary units.
c Mean ± S.E. of 3 measurements.
near that measured for clone C6, and all had approximately twice the DNA content of the smaller clones (not shown). Significant variation in TSP-180 expression, however, was also found among clones with similarly sized cells (Table 1; Chart 2B), indicating that cell size cannot account for all clonal variation.

To explore whether clonal TSP-180 differences were associated with a general variation of surface antigen expression, another line 1 surface antigen, P-100, was evaluated using a specific monoclonal antibody and flow cytometry. Table 1 reveals that P-100 variation among these clones correlates with cell size, and that among similarly sized clones, P-100 differences are not large. Thus, differences in TSP-180 expression present among clones with similarly sized cells are not caused by some factor which affects the level of all surface antigens.

We found that TSP-180 expression in line 1 is cell cycle dependent, with G2 cells having approximately 1.7 times as much TSP-180 as cells in G1 (Chart 3). Similar results were obtained with the 6 representative clones listed in Table 1. For this to generate clonal TSP-180 variation, clones would have to differ with respect to cell cycle phase composition. When cell cycle analysis of these clones was performed, however, no differences in distribution were found (41% in G1, 47% in S, and 12% in G2-M). Thus, one can conclude that the differences in TSP-180 expression found among the clones described in Table 1 are not cell cycle related.

Antibody and Complement-mediated Lysis. To ascertain whether the differences in TSP-180 expression among the clones listed in Table 1 were of sufficient magnitude to have functional consequence, we looked at cell viability following TSP-180-directed antibody and complement treatment (Table 2). To control for factors besides antigen density that potentially could affect antibody-mediated lysis (21), we also examined P-100-related complement-induced lysis, since P-100 density is similar for all of these clones (Table 1). Variation in lysis susceptibility among clones A11, C10, C25, and A13 appears totally dependent on differences in TSP-180 density, since similar levels of P-100-related lysis were observed for all. Clones C10 and A5, however, have a reduced susceptibility to both TSP-180- and P-100-related lysis. The above demonstrates that these differences in clonal TSP-180 expression are large enough to produce differential effects, and can influence the percentage of cells able to escape from TSP-180-related antibody and complement-mediated lysis.

Intraclonal Heterogeneity. To eliminate the remote possibility that intraclonal heterogeneity represents extensive heritable variation, 4 line 1 clones were subcloned (Table 3). As expected, most limiting dilution subclones did not significantly differ in TSP-180 expression from their respective parent clone upon evaluation approximately 2 weeks after cloning. Furthermore, variations in TSP-180 expression among subclones from any clone were small enough so that TSP-180 differences initially present among the clones were, for the most part, also maintained by the subclones. However, 2 of the 48 subclones screened, and 1 of 32 line 1 clones evaluated, were significantly different (at the 0.01 level) from a parent in expression of TSP-180. Based on cell size and DNA content (not shown), the highest TSP-180-expressing line 1 and C10-derived clones were near tetraploid, not an uncommon finding (3), and are similar therefore to the larger cell-sized clones described previously. Although a small amount of heritable TSP-180-related heterogeneity, possibly comparable to that present in line 1, can therefore be detected in some clones as soon as 2 weeks after cloning, most intraclonal expression variation is not heritable.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Anti-TSP-180 (1:200)</th>
<th>Anti-TSP-180 (1:1000)</th>
<th>Anti-P-100 (1:200)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A11</td>
<td>52</td>
<td>33</td>
<td>53</td>
</tr>
<tr>
<td>C10</td>
<td>30</td>
<td>21</td>
<td>40</td>
</tr>
<tr>
<td>A5</td>
<td>23</td>
<td>10</td>
<td>42</td>
</tr>
<tr>
<td>C6</td>
<td>57</td>
<td>54</td>
<td>53</td>
</tr>
<tr>
<td>C25</td>
<td>23</td>
<td>18</td>
<td>54</td>
</tr>
<tr>
<td>A13</td>
<td>29</td>
<td>11</td>
<td>51</td>
</tr>
</tbody>
</table>

*Percentage of dead following 2-step antibody and complement treatment minus percentage of dead after treatment with one antibody and complement (typically about 20%).

A 1:200 dilution of either antibody is saturating.
Table 3

Subclone variation of TSP-180 expression

Randomly selected clones were evaluated using flow cytometry approximately 2 weeks after cloning. Shown are data relating to 32 line 1 clones, 22 clones from line 1 after it had been cultured 13 months, and 12 subclones each of freshly thawed clones A11, C10, C6, and A13. For each cell line, the mean TSP-180 fluorescence values of the highest and lowest expressing clones are given (range), and the average (± S.D.) of all mean TSP-180 values is indicated, along with the uncloned level of the line. All fluorescence distributions had similar shapes and CVs.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mean fluorescence channel</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultured parent</td>
<td>156 ± 75</td>
<td>84 ± 5 (6)</td>
</tr>
<tr>
<td>Clone A11</td>
<td>125 - 99</td>
<td>111 ± 8 (7)</td>
</tr>
<tr>
<td>Clone C10</td>
<td>179 - 91</td>
<td>99 ± 4 (4)</td>
</tr>
<tr>
<td>Clone C6</td>
<td>222 - 194</td>
<td>202 ± 10 (5)</td>
</tr>
<tr>
<td>Clone A13</td>
<td>80 - 56</td>
<td>76 ± 6 (6)</td>
</tr>
</tbody>
</table>

Average of three mean fluorescence values made 2 days apart referenced to 50th bright fluorospheres, mean fluorescence channel 167. The difference associated with a TSP-180 value averaged ± 2% for the 122 clones analyzed.

The difference from the average is significant (p < 0.01; Student’s t test).

The highest TSP-180-expressing clone was excluded from this calculation to make the S.D. reflective of the variation in mean TSP-180 expression present among the other analyzed clones.

Table 4

Stability of antigen expression following extensive propagation in vitro

Cell lines were propagated in vitro for 5 months (approximately 45 passages) and then compared to freshly thawed cryopreserved stocks for expression of TSP-180 and P-100 using a flow cytometer (the parent line was propagated for 10 months).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% of change in mean expression a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone A11</td>
<td>−15 ± 5</td>
</tr>
<tr>
<td>Clone C10</td>
<td>−12 ± 5</td>
</tr>
<tr>
<td>Clone A5</td>
<td>−15 ± 6</td>
</tr>
<tr>
<td>Clone A6</td>
<td>−10 ± 12</td>
</tr>
<tr>
<td>Clone C25</td>
<td>−13 ± 7</td>
</tr>
<tr>
<td>Clone A13</td>
<td>−9 ± 9</td>
</tr>
<tr>
<td>Line 1</td>
<td>−26 ± 3</td>
</tr>
</tbody>
</table>

Average S.E. of change of 4 measurements made on separate days.

Average of three mean fluorescence values made 2 days apart referenced to 50th bright fluorospheres, mean fluorescence channel 167. The difference associated with a TSP-180 value averaged ± 2% for the 122 clones analyzed.

The difference from the average is significant (p < 0.01; Student’s t test).

The highest TSP-180-expressing clone was excluded from this calculation to make the S.D. reflective of the variation in mean TSP-180 expression present among the other analyzed clones.

Stability of Antigen Expression and Heterogeneity.

Reproducible changes in TSP-180 expression accompanied long-term cell passage of clones A11, C10, A5, and C25 as well as the parent, while only clone A11 and the parent displayed different mean P-100 values (Table 4). These perturbations were, however, not large enough to eliminate differences in TSP-180 expression initially present among these clones. TSP-180 levels decreased without FALS or P-100 expression changing for some clones (C10, A5, C25), and even though parental P-100 values increased, indicating that a trivial or artifactual explanation of culture-related TSP-180 change is unlikely.

Changes in TSP-180 expression we observed were not associated with any significant histogram broadening or bimodality (Chart 4), which suggested that no large increase in TSP-180-related heterogeneity occurred following long-term passage of either line 1 or the cloned lines. To further verify this finding, TSP-180 variation among clones derived from original line 1 stock and long-term passed line 1 was compared (Table 3). The S.D. entry indicates that 13 months of in vitro passage just slightly increased the probability of isolating clones which differed in surface expressed TSP-180. Line 1 TSP-180 heterogeneity thus appears to be quite stable during in vitro passage.

Determinants of Nonheritable Antigenic Heterogeneity.

Since cells in G2 display on the average more surface-expressed TSP-180 than cells in G1 (Chart 3), one can conclude that the cell cycle-dependent expression of TSP-180 is in part responsible for some nonheritable variation. To investigate whether TSP-180 expression is related to cell size, correlated measurements of TSP-180 and either FALS or TF were made (Chart 5). The bivariate displays reveal that surface TSP-180 is indeed somewhat dependent on cell size (Chart A5, and B), being most strongly correlated with TF, which is proportional to cell surface area (Chart 5C). However, even for cells displaying similar TF values, considerable TSP-180 heterogeneity is still present, indicating that not all nonheritable TSP-180 variation is cell size related.

Since G2 cells are typically larger than cells in G1 (mean volumes differ by approximately 2-fold), the cell cycle-dependent expression of TSP-180 would necessitate TSP-180 being somewhat correlated with cell size, as we have found. To investigate whether TSP-180 exhibits any relationship to cell size in the absence of a cell cycle effect, TSP-180, DNA, and TF list mode data were obtained from appropriately double labeled line 1 cells and, subsequently, fluorescence DNA gates were used to evaluate cells only in G1 or G2. Although cells labeled with both FITC and PI are ethanol-fixed and are overall slightly smaller in diameter than unfixed cells (Chart SD), the relationship between TSP-180 and TF appears essentially unchanged by fixation. However, much less if any correlation between TSP-180 and TF is evident for cells in G1 or G2 (Chart 5C, and F). Clearly, substantial TSP-180 heterogeneity is present within G1 or G2-restricted line 1 populations unrelated to cell size variation.

DISCUSSION

In the present study, we examined the cell surface expression of a M180,000 TAAg (TSP-180) within the noninduced BALB/c...
l lung carcinoma line 1. Monoclonal antibodies, immunofluorescence, and flow cytometry were used to quantify surface-expressed TSP-180. We demonstrated that essentially all line 1 cells express TSP-180 above background levels, but that TSP-180 expression is quite heterogeneous. Furthermore, cells of a defined size and cell cycle stage still exhibited considerable heterogeneity of expression. Although variable surface antigen expression in tumor cell populations is not unique to line 1 or TSP-180, e.g., Burchiel et al. (4), also using flow cytometry, recently reported on the heterogeneous expression of TAAg by human melanoma lines, the heritable basis of most antigenic variation has been largely unexplored.

If one randomly samples a heterogeneous cell population by cloning, heritable variation can be distinguished from nonheritable heterogeneity. A large component of heritable antigenic heterogeneity would be indicated by frequently observing clones displaying sizeable differences in mean expression, each having less-associated heterogeneity than found in the parent line. Nonheritable heterogeneity is that which can be generated by single cells before heritable variants arise. Recently derived clones, therefore, represent populations displaying minimal heritable heterogeneity.

With flow cytometry, all analyzed limiting dilution line 1 clones were found to have as much heterogeneity as observed in the parent, suggesting most TSP-180 variation in line 1 is nonheritable. Recloning select clones formally established that most were still very homogeneous after 2 weeks of growth, and that intraclonal heterogeneity revealed with flow cytometry did not result in detectable variation. The vast majority of 263 clones we evaluated also expressed TSP-180 at levels indistinguishable from the parent, further indicating a relative absence of heritable variation within line 1. A small component of heritable heterogeneity was clearly demonstrated, however, by the occasional isolation of clones able to maintain large differences in surface-expressed TSP-180. The minor contribution TSP-180 variant clones make to the line 1 population apparently cannot be detected with flow cytometry. That is, TSP-180 heterogeneity detected with flow cytometry represents only nonheritable variation. Experiments performed mixing together clones C10 and C6, which differed by approximately 2-fold in average TSP-180 expression, indicated that either clone had to comprise roughly 5% of the mixed cell population before it could be reliably identified using flow cytometry (data not shown).

Heritable variation of surface expressed TSP-180 could have a genetic and/or epigenetic basis. Further studies are needed to determine how differential TSP-180 expression among clones is regulated. In this report, we did demonstrate, however, that clones differing in TSP-180 expression don't necessarily differ in cell size, cell cycle distribution, or expression of P-100, another line 1 surface protein. In particular, finding clones with different amounts of TSP-180 but similar levels of P-100 makes it unlikely that clonal variation of TSP-180 expression could be the result of a surface antigen masking effect.

Similar to the results of Harris et al. (11), who explored the generation of heritable tumor variants with high metastatic potential in the mouse KHT tumor, we observed that heterogeneity quickly develops in cloned line 1 cell populations. Heritable TSP-180 variants were isolated by subcloning 4 cloned lines after approximately 5 \times 10^7 cells had been generated (approximately 2 weeks). Despite the initial presence of variants, TSP-180 heterogeneity, assessed by both quantitative fluorescence and cloning, appeared stable throughout passage. Our results suggest that TSP-180 heterogeneity develops in cloned line 1 populations until it reaches a steady-state level typified by the line 1 parent. At that point, TSP-180 variant generation is either balanced by loss or somehow suppressed, since clones appreciably different from the parent are rarely isolated even after 13 months of culture. Long-term in vitro stability of heterogeneity has also been observed by Poste et al. (22) and Chow and Greenberg (7), using for phenotypic markers metastatic potential and antibody-mediated lysis, respectively.

Not all tumor surface antigen heterogeneity reported in the literature has been found to be stable during in vitro passage. Leibson et al. (16) and Yeh et al. (28) both observed the appearance of antigen-negative variants using mouse and human myeloma systems, respectively. Even though average TSP-180 expression decreased by 25% after 13 months of passage, neither flow cytometry nor cloning revealed the presence of a
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stable TSP-180-negative subline. In addition, all of 16 clones, obtained by sorting single TSP-180-stained cells, which fluoresced near control levels, directly into individual cloning wells, were TSP-180-positive after 2 weeks of growth. One possible explanation for not detecting a TSP-180-negative heritable phenotype is that a gene essential for surface TSP-180 expression is located near a locus controlling cell growth. Analogous to TAAg-negative variant selection, immune system-related pressure could nevertheless select for a dominant heterogeneous subpopulation displaying less overall TAAg (20). Distinguishing escape related to lowered TAAg expression from that caused by loss or absent TAAg could be important when considering the therapeutic use of substances, such as interferon, which may modulate tumor antigen levels (18).

Findings that tumor progression (27) or metastatic potential (1, 2) can correlate with an apparent lack of tumor antigen suggest that heritable differences in TAAg expression among tumor cells could have biological significance. Perhaps nonheritable TAAg heterogeneity could also influence tumor growth in vivo, provided TAAg levels don’t increase rapidly relative to cell division. Differential immune system recognition of the clones detailed in this report is suggested by our observation relating TSP-180 expression to the percentage of cells lysed following TSP-180-specific antibody and complement treatment. Studies using these clones directed at determining whether TSP-180 expression is associated with line 1 growth in vivo are currently in progress.

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Heterogeneity and Clonal Variation Related to Cell Surface Expression of a Mouse Lung Tumor-associated Antigen Quantified Using Flow Cytometry


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