A New Murine Model System for the in Vitro Development of Thymoma Cell Heterogeneity

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ABSTRACT

We have established and characterized a continuous T-cell line derived from the bone marrow of an AKR mouse with disseminated lymphoma. The original tumor cell line is heterogeneous with respect to several markers of thymocyte differentiation. Clones from the line differ in the expression of Thy-1, Pgp-1, and H-2Kk surface antigens. These clones also differ in their sensitivity to glucocorticoid-induced cell lysis. The quantity, affinity, and nuclear translocation properties of the glucocorticoid receptor are similar in the hormone-sensitive and -resistant clones. Furthermore, dexamethasone-resistant T-cells can be selected in vitro from freshly cloned cells sensitive to hormone-induced lysis at high frequency and without mutagenesis. Of several randomly sampled, spontaneously arising, independently derived dexamethasone-resistant clones, all show a coordinate reduction in cell surface Thy-1 and ThB expression with no detectable changes in glucocorticoid receptor properties. Following treatment with the DNA-demethylating agent 5-azacytidine, the original dexamethasone-resistant T-cell line is as resistant as the dexamethasone-resistant derivatives obtained in vitro in regaining sensitivity to lysis. These results collectively suggest a role of DNA methylation in hormone resistance and are consistent with a model of thymocyte differentiation in which a glucocorticoid-sensitive cell is the progenitor of hormone-resistant T-cells.

INTRODUCTION

Glucocorticoid hormones elicit a cytolytic response in cortical thymocytes and some T-lymphoma cell lines (7, 32). The cytolytic response is one basis for the therapeutic use of glucocorticoids in several types of leukemia. The hormone response is receptor mediated (44, 41, 35, 31), and defects in receptor-hormone binding or receptor-hormone translocation to the nucleus block the lytic response (2, 36). All glucocorticoid-resistant variants described previously obtained from sensitive cell lines in vitro have been resistant due to defects in glucocorticoid receptor function (24, 18). However, receptor-containing noncortical thymocytes, and functional peripheral T-cells are not lysed by glucocorticoids. Thus, either defects in hormone-receptor function, or the state of T-cell differentiation can determine resistance to lysis by glucocorticoids (2, 3). Furthermore, one established T-lymphoma cell line which contains functional glucocorticoid recep-

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cells using [3H]dexamethasone, as reported previously (1, 36). Assays were carried out in the presence or absence of a 200-fold excess of unlabeled dexamethasone to determine nonspecific dexamethasone binding, which was subtracted. Hormone-resistant cells were obtained from cloned hormone-sensitive cells by selection of 10⁶ cells in 1 μM dexamethasone at either 1 x 10⁶ or 5 x 10⁵ cells/well in 24-well trays.

Surface Antigen Analysis. Surface antigen expression was measured quantitatively by flow cytometry. Samples were stained and analyzed on the Salk Institute flow microfluorimeter as reported previously (26). Briefly, 5 x 10⁶ cells were incubated with undiluted hybridoma tissue culture supernatants for 30 min on ice, washed, and subsequently incubated with fluorescein isothiocyanate-conjugated goat anti-rat or goat anti-mouse IgG. Cells were washed and suspended in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered DMEM containing 5% fetal bovine serum. Background samples were subtracted for hybridoma tissue culture supernatants. Analysis of flow cytometry data was performed using a modified DISP-8 computer program (40).

Measurement of Cellular DNA Content. Exponentially growing cells (5 x 10⁶) were washed in Ca²⁺- and Mg²⁺-free phosphate-buffered saline and fixed in 70% methanol. The cells were stained with mithramycin (10 μg/ml) in 25% methanol and analyzed within 2 hr by flow cytometry (4).

5-Azacytidine Treatment. On the basis of a dose-response curve, which was conducted in the range of 0.1 to 5 μM 5-azacytidine, 0.4 or 1 μM concentrations of the drug were used, because they were found to slow the growth of SL12 clones with a minimal amount of cell death (17, 27). Exponentially growing cells were plated for 16 hr in complete medium at 2 to 5 x 10⁵ cells/ml in freshly prepared 5-azacytidine. The cells were washed 3 times in DMEM and plated at 10⁵ cells/ml. After 48 hr (2 to 3 cell doublings), the populations were tested directly for dexamethasone sensitivity and cloned by limiting dilution, and the clones were tested for dexamethasone sensitivity.

Materials. Mithramycin and dexamethasone were obtained from Sigma Chemical Co., St. Louis, MO; 5-azacytidine from Calbiochem, La Jolla, CA; [3H]dexamethasone from Amersham/Seattel Corp., Arlington Heights, IL; and fluorescein isothiocyanate-conjugated antiserum from Antibodies, Inc., Davis, CA, or Cappel, Inc., Westchester, PA.

RESULTS

Characterization of the Glucocorticoid Response. The T-lymphoma cell line SL12 was tested for glucocorticoid sensitivity by measuring cell growth in the presence and absence of 1 μM dexamethasone. The results suggested that the cell line was heterogeneous with respect to hormone responsiveness. Therefore, SL12 was cloned by limiting dilution, and several clones were tested for glucocorticoid sensitivity. Two clones, SL12.1 and SL12.4, are strikingly different in their hormone response. Chart 1A shows the growth response of the 2 clones in 1 μM dexamethasone.

Since the dexamethasone-induced cytolytic response is receptor mediated, we measured dexamethasone-binding activity (2) and dexamethasone-receptor nuclear translocation (36) in the 2 cloned lines. Chart 1B shows a Scatchard analysis of specific [3H]dexamethasone binding. Both the dexamethasone-sensitive and dexamethasone-resistant T-cell lines contain approximately 30,000 specific binding sites per cell. This value is quite similar to the receptor content of the well-characterized dexamethasone-sensitive lymphoma cell line WEHI-7 (1) and the dexamethasone-resistant AKR-derived cell line SAK-8 (17). In addition, the binding affinities (Kd) and nuclear translocation properties (36) of the dexamethasone-receptor complex reveal similar values for the SL12.1 dexamethasone-resistant and SL12.4 dexamethasone-sensitive cell lines (Table 1). Thus, the biochemical properties of the hormone receptor are similar in both the dexamethasone-sensitive and dexamethasone-resistant T-cell lines, suggesting that the dexamethasone resistance is not due to defects in hormone-receptor function.

Since the hormone-response depends on functional hormone-receptor and at least one other unlinked locus which determines the lysis function (17), we tested the possibility that the dexamethasone resistance of SL12.1 arose from chromosome loss. The DNA content of the SL12 dexamethasone-sensitive and dexamethasone-resistant T-cell lines were compared by flow cytometry with cells which contain the normal diploid complement of 40 chromosomes (1). All 3 cell lines have indistinguishable DNA contents suggesting the the SL12.1 dexamethasone-resistant T-cell line has not lost a significant amount of chromosomal material (data not shown).

Selection of Hormone-resistant Clones from the Hormone-Sensitive SL12.4. It is not known whether the heterogeneity of the SL12 cell line occurred subsequent to a single transformation.
event or, alternatively, whether the 2 cell types resulted from independent tumors occurring in the animal prior to the establishment of the cultured cell line. To investigate this point, we used hormone selection in an attempt to isolate “SL12.1-like” hormone-resistant cells which may be present among the hormone-sensitive SL12.4 cloned population. Specifically, it is possible to use dexamethasone cytolytic selection on the dexamethasone-sensitive cells to determine whether the hormone-sensitive cell line can give rise to dexamethasone resistance without mutagenesis. In the first experiment, 10^6 SL12.4 dexamethasone-sensitive cells were placed in multitest wells at 5 x 10^6 cells/well in the presence of 1 μM dexamethasone for 2 weeks. Surprisingly, approximately 50% of the wells contained living cells. The survivors were cloned by limiting dilution in the absence of dexamethasone. Among the 48 clones retested for dexamethasone resistance, 3 distinct phenotypes were obtained. The growth response in 1 μM dexamethasone of representatives of each type are shown in Chart 2. RS4.1 is fully resistant in that it grows at the same rate in the presence and absence of hormone and lacks any measurable hormone-receptor binding (Table 1). In contrast, RS4.3 grows more slowly in dexamethasone, and RS4.2 is growth arrested by dexamethasone, but neither is lysed. Cells with the latter 2 phenotypes have levels of hormone binding and nuclear translocation quantitatively similar to the dexamethasone-sensitive parent SL12.4 (Table 1).

To verify and extend the finding that dexamethasone-resistant T-cells can be derived from the dexamethasone-sensitive SL12.4 line, the cells were recloned, and 15 subclones, shown to be dexamethasone-sensitive in growth assays, were independently subjected to hormone selection. Both dexamethasone-resistant and dexamethasone-growth-arrested cells were obtained from each of the 15 subclones following dexamethasone selection. Of the 67 hormone-resistant and 84 growth-arrested clones examined, all were shown to have unaltered dexamethasone binding and hormone-receptor nuclear translocation properties (data not shown).

In several independent selection experiments, more than 5 x 10^6 dexamethasone-sensitive SL12.4 cells and freshly obtained SL12.4 subclones were subjected to lytic selection by 1 μM dexamethasone. Only 2 clones have been isolated which are fully resistant and lack hormone binding. These rare variants are likely to be the result of spontaneous mutations (24). When receptor-defective cells occur, they are easily recovered because they have a marked growth advantage in the presence of hormone. The frequency of the receptor-positive resistant and growth-arrested phenotypes has been difficult to establish because they occur in each selection experiment as mixtures. Neither type grows extremely well in hormone-selective medium; the growth arrested type divides only once or twice, whereas the dexamethasone-resistant type, which shows unaltered receptor-binding properties, grows to a saturation density of only 10^6 cells/ml. However, in several independent experiments, we have estimated a frequency of between 5 x 10^-3 and 5 x 10^-4 orders of magnitude higher than the spontaneous frequency of receptor mutations (24). In order to determine if the spontaneously occurring resistant and growth-arrested derivatives are stable, we have tested 365 subclones from 4 independently derived growth-arrested and resistant lines for hormone-sensitivity, and none were sensitive.

The DNA content of representatives of the 3 phenotypes was measured. Each had the same patterns on a DNA histogram as their dexamethasone-sensitive parent indicating no significant losses in DNA content (data not shown).

5-Azacytidine Effects on Hormone Response. The cytidine analogue 5-azacytidine has been used to distinguish between mutations and epigenetic events occurring in cell lines (21). 5-Azacytidine is an unmethylatable analogue of cytidine that also inactivates nuclear pools of the enzyme hemimethyltransferase, which is responsible for the maintenance of established DNA methylation patterns (5, 6, 11). Drug treatment results in a massive demethylation of cytidine residues in cellular DNA up to 90% under some conditions (5). Hypomethylation has been associated with active gene expression (9, 20), whereas hypermethylation of cytidine residues is frequently associated with genes in their inactive conformation (8, 21, 45, 46, 50, 53).

Previous work with the AKR SAK-8 dexamethasone-resistant T-cell line revealed that the lytic function of hormone-resistant T-cells containing functional receptor can be activated upon treatment with 5-azacytidine (19). In this study, the original cloned tumor cell line which is dexamethasone resistant (SL12.1) and a representative from each class of the dexamethasone-resistant derivatives obtained from the dexamethasone-sensitive SL12.4 line were treated with 5-azacytidine to determine whether the resistance was due to a mutation in some step of the hormone response beyond hormone-receptor complex translocation to the nucleus.

Both the 5-azacytidine-treated and untreated populations of SL12.1 were cloned by limiting dilution, and 48 clones were tested for growth in 1 μM dexamethasone. Of the 5-azacytidine derived clones, 8 of 48 are sensitive to dexamethasone-induced lysis. Chart 3A shows the growth properties of one such clone.

Chart 2. Glucocorticoid sensitivity of derivatives of the SL12.4 cell line. Cloned cell lines were grown in the absence (○) or presence (△, ▲) of 1 μM dexamethasone, and living cells were counted at daily intervals. ○, growth of all cell lines in the absence of dexamethasone; ▲, SL12.4 parental cell line; △, RS4.1 receptorless cells; ▲, RS4.3 dexamethasone-resistant T-cell line; △, RS4.2 dexamethasone growth-arrested cell line.
Thymoma Cell Heterogeneity

Not be rendered sensitive by 5-azacytidine. As expected, no dexamethasone-sensitive clones (0 of 48) were derived from receptor-defective RS4.1 (Chart 3B), whereas drug treatment produced full dexamethasone sensitivity in 3 of 20 growth-arrested and 9 of 48 dexamethasone-resistant cell lines (Chart 3, C and D; Table 1). The hormone sensitivity observed in approximately 15% of clones from SL12.1, RS4.2, and RS4.3 results from 5-azacytidine treatment, as none of the clones (0 of 158) tested from the untreated cells were sensitive to dexamethasone-induced lysis. Both the original dexamethasone-resistant SL12.1 tumor clone and the growth-arrested and resistant receptor-containing derivative phenotypes can be rendered sensitive to hormone-induced cytolysis following 5-azacytidine treatment. Dexamethasone-sensitive clones obtained by 5-azacytidine treatment of hormone-resistant cells vary in their stability. Most of the clones rapidly accumulate resistant cells, becoming entirely resistant to lysis within 2 to 3 months. Two clones have remained completely sensitive after 6 months of continuous culture. The reacquisition of resistance may result from remethylation similar to that observed in SAK-8 cells (19) and argues against chromosomal breaks or rearrangements.

Characterization of Surface Markers. To further compare the original tumor clones SL12.1 and SL12.4 with the derivatives obtained in vitro, we analyzed the expression of 8 surface antigens commonly found on T-cells, thymocytes, and cell lines derived from this lineage. The dexamethasone-resistant SL12.1 cell line displays Thy-1, T200, TL, Pgp-1, and H-2Kk and has no detectable ThB, Lyt-1, or Lyt-2 expressed on the cell surface (data not shown). In contrast, dexamethasone-sensitive SL12.4

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lacks detectable H-2K\(^\alpha\), Pgp-1, Lyt-1, and Lyt-2 (data not shown) but is ThB positive and expresses Thy-1, TL, and T200 (Chart 4). It is quite evident that SL12.1 and SL12.4 differ not only in their hormone responsiveness but also in their surface antigen characteristics.

The dexamethasone-resistant derivatives of hormone-sensitive SL12.4 were examined for their surface antigen expression (Chart 4). The receptorless derivative expresses the same surface antigen pattern as does the parental SL12.4 cell line. In contrast, both the growth-arrested RS4.2 and the resistant RS4.3 derivatives show a 5-fold lower expression of Thy-1 and a 2- to 3-fold lower expression of ThB, whereas T200 remains essentially unchanged from the parental SL12.4 cell line (Table 2). To confirm the coordinacy of the change in hormone response and surface antigen expression shown in Chart 4, several independently isolated growth-arrested and resistant clones were isolated from 3 subclones of SL12.4 and examined for surface antigen expression. In all cases, T200 expression showed little or no significant change. In every case examined, the hormone selected subclones which had hormone-receptor binding, showed a 3- to 6-fold lower Thy-1 expression and a 2- to 3-fold lower ThB expression, as compared to their respective parental clone. The cell surface antigen patterns consistently show a reduction in Thy-1 and ThB expression which is coordinate with changes in hormone sensitivity.

Although SL12.4 sensitive cells do give rise to hormone-resistant cells similar to SL12.1, the surface antigens expressed by the derivative are not identical. In particular, SL12.1 expresses high levels of Pgp-1 and H-2K\(^\alpha\), whereas the derivatives of SL12.4 lack detectable levels of both surface antigens. Therefore, we conclude that the heterogeneity observed in the original tumor cell lines is different from the heterogeneity generated in the in vitro selection experiments, and thus, no SL12.1-like cells were selected in vitro.

5-Azacytidine Effects on Surface Antigen Expression. A number of hormone-sensitive clones obtained following 5-azacytidine treatment of hormone-resistant and growth-arrested cells were examined for possible changes in surface antigen expression of ThB, Thy-1, and T200. No significant changes in surface antigen expression were observed in any of the 5-azacytidine-treated clones examined (Chart 5). Furthermore, surface antigen expression in several 5-azacytidine-treated populations from independently derived hormone-resistant cell lines were examined. If only 5% of the treated cells had reexpressed original levels of Thy-1 or ThB, the flow cytometry analysis could easily detect it. However, no evidence for reactivation of surface antigen expression was obtained (data not shown). Finally, in 3 attempts to isolate cells which reexpress parental levels of Thy-1 following 5-azacytidine treatment, fluorescence-activated cell sorting was used. Parallel reconstruction experiments showed that at mixtures of 1:10,000, Thy-1-bright, dexamethasone-sensitive parental cells could be isolated from at 10,000-fold excess of Thy-1-negative derivatives (23). After 2 cycles of cell sorting following treatment with 5-azacytidine, flow cytometry revealed no significant increase in Thy-1 or ThB expression (data not shown). The 5-azacytidine-treated cells which had been selected by 2 cycles of fluorescence-activated cell sorting were cloned and subsequently tested for glucocorticoid sensitivity and surface antigen expression. Only 15% of those clones were hormone sensitive, a frequency similar to that obtained in a number of other experiments wherein no cell sorting was used. Furthermore, these sensitive clones, none showed significant changes in Thy-1, ThB, or T200 expression. Therefore, we conclude that 5-azacytidine does not reactivate parental levels of either Thy-1 or ThB expression under experimental conditions in which 15%

![Chart 5](chart5.png)

**Chart 5.** Surface antigen expression following 5-azacytidine treatment. The SL12.4 parental dexamethasone-sensitive (DEX\(^5\)) cell line, an independently derived dexamethasone growth-arrested (DEX\(^{10a}\)) derivative, and a hormone-sensitive clone following 5-azacytidine (Aza-C) treatment were examined for the expression of ThB, Thy-1, and T200 and were analyzed as in Chart 4.
of the cells have reactivated a function responsible for hormone-induced cell lysis.

DISCUSSION

We have isolated and characterized the SL12 T-lymphoma cell line which displays marked heterogeneity with respect to surface antigen expression and glucocorticoid responsiveness. The SL12.4 clone gives rise, at high frequency (1 in 10³ to 3 in 10⁶) and without mutagenesis, to cells which have coordinately changed the expression of 3 markers typical of cortical thymocytes (hormone response, Thy-1, and ThB). When receptor-containing cells resistant to hormone-induced lysis are treated with the DNA demethylating agent 5-azacytidine, approximately 15% of the clones obtained are fully sensitive to lysis by dexamethasone. This succession of events is illustrated in the following flow chart.

To our knowledge, no other hormone-sensitive cell line has been described which spontaneously gives rise to hormone-resistant cells with apparently functional hormone receptors. The 5-azacytidine experiments suggest that methylation might inactivate a function necessary for lysis in receptor-containing resistant cells. However, other possible actions of 5-azacytidine such as chromosomal breaks or rearrangements, although unlikely because of the reversibility of the phenotype and the high frequency of sensitivity, have not been excluded. The lytic response expressed by glucocorticoid sensitive cells has been shown to be a dominant genetic characteristic (1, 2, 17, 18, 35, 37, 44) in a number of different somatic cell hybrids. Furthermore, a lytic function has been activated in resistant cells treated with 5-azacytidine and which exhibit lower amounts of cellular deoxymethylcytosine (19). It seems probable, therefore, that the lysis function is expressed in sensitive cells and is inactive in resistant cells.

Of the 3 coordinate changes observed, only the hormone response can be reactivated by 5-azacytidine. DNA methylation might merely maintain, but not initiate, the development of hormone resistance, whereas the regulation of the surface antigen expression may not directly involve DNA methylation. Perhaps a controlling regulatory gene coordinately initiates the inactivation of the lytic function and the reduced expression of the 2 surface antigens. Clarification of the mechanisms which trigger the coordinate changes and/or which regulate their expression will be helpful and stimulating discussions; Joseph Trotter for invaluable help with flow cytometry computer programs; Steven Mann for help with the dexamethasone selection experiments; and Scott Glaser for his contribution to the experiments measuring the effect of 5-azacytidine on surface antigen expression.

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