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Glucocorticoid-Resistant Lymphoma Cell Variants That Contain Functional Glucocorticoid Receptors

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A mouse T-lymphosarcoma cell line stably infected with mouse mammary tumor virus (MMTV) was used as the parent line for a genetic analysis of two glucocorticoid hormone responses, hormone-induced cytolysis and stimulation of viral gene expression. Variants were selected for survival and elevated expression of MMTV proteins in the presence of the steroid. The MMTV marker provided a sensitive test for glucocorticoid receptor (GR) function in the hormone-resistant variants. This strategy resulted in the isolation of two novel types of hormone-resistant variants. One type of variant with only about 25% of the level of GR found in the parent line was resistant to the cytolytic effects of glucocorticoid but produced increased levels of MMTV gene products in response to the hormone. This variant phenotype demonstrated that the MMTV response requires fewer GR than the cytolytic response. Another variant, which required approximately 100-fold higher concentrations of hormone than the wild-type cells for both responses, apparently contained GR with altered hormone-binding properties.

Many thymus-derived lymphoma cell lines exhibit a dramatic response to physiological concentrations of glucocorticoid hormones that includes cessation of proliferation within a few hours and cytolysis after 1 to 3 days of continuous exposure (12, 14, 17). The cytolytic response has provided the basis for a genetic analysis of steroid hormone action via the selection of cell variants that continue to grow in the presence of the hormone. A large number of glucocorticoid-resistant variants have been analyzed in the mouse S49, mouse WEHI7, and human CEM-C7 T-lymphosarcoma cell lines (11, 20, 23, 30). Almost all of these variants have been shown to have either reduced levels or physically altered forms of the glucocorticoid receptor (GR) protein (reviewed in reference 15). These variants have proven to be extremely valuable for defining the structural and functional properties of the GR protein.

The largest number of resistant lines have no measurable glucocorticoid-binding activity; a smaller group has less than about 25% of the wild-type binding activity. These two groups, designated receptor negative (r^-), provided crucial genetic evidence that the cytolytic response (24) and the stimulation of mouse mammary tumor virus (MMTV) gene expression (9) are mediated by the GR. Three other phenotypically distinct types of inactive GR mutants were identified: nuclear transfer-deficient (nt^-) receptors; nuclear transfer-increased (nt^i) receptors (30); and activation-labile (act^l) receptors with temperature-sensitive hormone-binding capacity (10). Recent mapping studies of GR mutations in some of these variants have helped to begin the process of defining the functional domains of the GR (4, 18, 19).

Analysis of the hormone-resistant variants and somatic cell hybrids constructed with various quantities of GR (2, 7) indicated that the concentration of glucocorticoid hormones required to evoke the cytolytic response bears an inverse relationship to the number of functional GR per cell. Furthermore, there appears to be a threshhold level of receptors (about 25% of the wild-type level in WEHI7 cells) below which there is no growth inhibition at any concentration of hormone. In hormone-resistant variants that retain low levels of hormone-binding activity, it has never been determined whether the residual hormone-binding activity represents functional GR and whether all glucocorticoid responses are similarly dependent on the level of GR in the cell.

In an effort to obtain novel types of hormone-resistant variants and to investigate further the relationship between GR levels and hormone responsiveness, we have devised a new strategy for selecting variants from cell line W7MG1, an MMTV-infected T-lymphosarcoma line derived from WEHI7. W7MG1 cells display at least three distinct glucocorticoid receptor-mediated responses: (i) growth inhibition and cytolysis; (ii) increased transcription of MMTV genes; and (iii) stimulation of the maturation of MMTV proteins (5, 21). We selected cells that were resistant to the cytolytic effects of glucocorticoids but retained the ability to express elevated levels of viral glycoproteins on the cell surface in response to glucocorticoids; our rationale for this strategy was that such variants should contain functional GR.

In this report we describe two new types of hormone-resistant variants resulting from the above selection scheme. Measurements of MMTV gene expression in these cells before and after administering hormone served as a sensitive functional test for GR. Examination of hormone dose-response relationships of the variants and physical and hormone-binding characteristics of the GR allowed us to demonstrate that one type of variant (W7M306a and W7M314a) had dramatically reduced levels of GR with apparently normal physical and functional properties; without the assays for MMTV gene expression, these variants would have been classified r^-. The second type of variant (W7M320b) contained a partially functional mutant receptor with a 100-fold reduction in hormone-binding affinity. The implications of our results are discussed with respect to the effect of GR levels on different hormone responses, the nature of the nuclear GR-binding sites associated with different responses, and the structure of the GR protein.

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MATERIALS AND METHODS

Materials. Unless otherwise indicated, the materials used in this study were those described previously (21). [^{3}H]dexamethasone ([^{3}H]dex) and [^{3}H]dexamethasone 21-mesylate were purchased from New England Nuclear Corp. Progesterone (Δ^4-pregnene-3,20-dione) and 17β-estradiol were purchased from Sigma Chemical Co.

Cell lines and genetic selections. The parental or wild-type cell line used for selection of variants was W7MG1 (5), an MMTV-infected subclone of mouse T-lymphosarcoma line WEHI7 (12). The selection and characterization of W7M302b, W7M326.4, and W7M329a have been described (21, 22). Variants W7M306a, W7M314a, and W7M320b (described here) were selected from mutagenized populations of W7MG1 cells by the same procedure (21). Briefly, cells were selected for survival in 10^{-6} M dex and for expression of MMTV proteins on the cell surface after growth in dex. Cell lines ADR6.M189D (19) and W7.418 (1) are dex-resistant sublines of WEHI7 with no hormone-binding activity. In addition, M189D cells contain no detectable immunoreactive GR protein. Procedures for cell growth, cloning, and counting for growth curves were described previously (5).

Quantitation of MMTV gene expression. Control cells or cells grown for 8 h with 10^{-6} M dex were analyzed for MMTV RNA and protein expression. Cytoplasmic MMTV RNA levels were measured as described previously (21) by cytoplasmic dot hybridization, with fixed cytosol from equivalent numbers of cells. Cell surface expression of MMTV proteins was detected by two methods (21): panning involved quantitation of cells binding to petri dishes coated with a monoclonal antibody against MMTV glycoprotein gp52; alternatively, the mean fluorescence was measured for cells incubated first with antiserum against gp52 or against total MMTV proteins and then with a secondary fluorescent antibody.

Uninfected cells (WEHI7) were included as negative controls in each of the assays. These cells produced no detectable signal in the cytoplasmic dot hybridization assay or the panning assay (21); the fluorescence signal for the uninfected cells was less than that for the W7MG1 cells grown without hormone (21) and was used to establish the background level of nonspecific fluorescence. W7M326.4, a previously characterized glucocorticoid-resistant cell line (derived from W7MG1) that lacks functional glucocorticoid receptors (nt^-), was previously established as a negative control for hormone responsiveness in each of the assays described above (21, 22).

Analysis of GR and their mRNA. For quantitation of GR mRNA, 6 μg of purified cytoplasmic RNA was applied to nitrocellulose (27) with a Slot-Blot apparatus (Schleicher & Schuell) and then hybridized with a labeled GR cDNA probe (18); the resulting autoradiogram was scanned with a densitometer to obtain quantitative data. Triplicate samples of two or three independent RNA preparations from each cell line were applied to the membrane. The mean value and standard deviation from the densitometer tracings for each cell line are expressed relative to the mean for W7MG1. The relative mean value for WEHI7 cells was 1.0 ± 0.3. Line M189D, which contains less than 5% of the wild-type level of GR mRNA by Northern blot analysis (18), served as a negative control. The sizes of the GR mRNAs were determined by blot hybridization analysis of electrophoretically resolved RNA preparations (4).

The amounts and sizes of GR protein species in the cell lines were determined by immunoblotting (3). Soluble proteins (150 μg) from each cell line were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose; GR on the filter was detected with a 1:500 dilution of an ascites preparation of monoclonal antibody IGR49 (29) and an alkaline phosphatase immunostain (Promega Biotec).

In most cases a whole-cell [^{3}H]dex-binding assay was used to determine the relative amount of hormone bound by variant and wild-type cells (6). Hormone bound by 10^7 cells was determined at 37°C at a dex concentration of 2×10^{-8}M. Data presented are the mean and standard deviation of two to three independent experiments, with each independent experiment consisting of triplicate determinations. The number of GR in W7MG1 cells was determined previously tobe 33,000 per cell (6). In addition, extracts from wholecells incubated with the affinity-labeled [^{3}H]dexamethasone 21-mesylate (5×10^{-8} M) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography to determine the size of the hormone-binding species (26). Relative binding of [^{3}H]dex in a cell extract was measured by a charcoal-binding assay (16), with a labeled hormone concentration of 5×10^{-8} M; the extract was prepared by freezing and thawing the cells in the specified buffer. The assay was performed in triplicate, and equivalent results were obtained in two independent experiments.

RESULTS

Dex-resistant variants with low levels of functional GR. The parental W7MG1 cell line was killed by concentrations of dex as low as 10^{-8} M (21) (Fig. 1A). In contrast, variants W7M306a and W7M314a were resistant to the presence of much higher concentrations: 306a cells grew normally in 10^{-5} M dex (Fig. 1B); the growth of variant W7M314a was inhibited by 10^{-7} M dex, but unlike W7MG1, the 314a cells survived for several days even in much higher concentrations of dex (Fig. 1C). Whole-cell [^{3}H]dex-binding studies with 2×10^{-8} M labeled hormone indicated that these variants contained only 20 to 30% of the specific hormone-binding activity found in W7MG1 cells (Table 1).

Analysis of immobilized cellular RNA by a quantitative blot hybridization method (RNA slot-blot hybridization) indicated that 306a and 314a cells contained about 30 to 40% as much GR-specific RNA as W7MG1 cells (Table 1). Blot hybridization analysis of electrophoretically separated RNA (Northern blots) gave approximately the same quantitative results and demonstrated that the transcripts in the variants were the same size (5 and 7 kilobases) as in the wild-type cells (data not shown).

When GR protein in the cells was analyzed by immunoblotting, a single 94,000-molecular-weight (94K) species was observed in all of the cell lines. Variants 306a and 314a contained approximately 20% as much GR protein as W7MG1 cells (Fig. 2 and Table 1). Essentially the same results were obtained when the cells were labeled with [^{3}H]dexamethasone 21-mesylate and the cell extract was examined by electrophoresis (data not shown). Thus, the glucocorticoid-binding activity and the GR RNA and protein species in 306a were 20 to 30% as abundant as in W7MG1 cells, and in 314a they were 20 to 40% as abundant as in the wild-type cells.

The degree of sensitivity to glucocorticoid-induced cytolysis is dependent on the number of GR per cell, and cells with less than about 25% of wild-type levels of GR are invariably glucocorticoid resistant (reviewed in reference

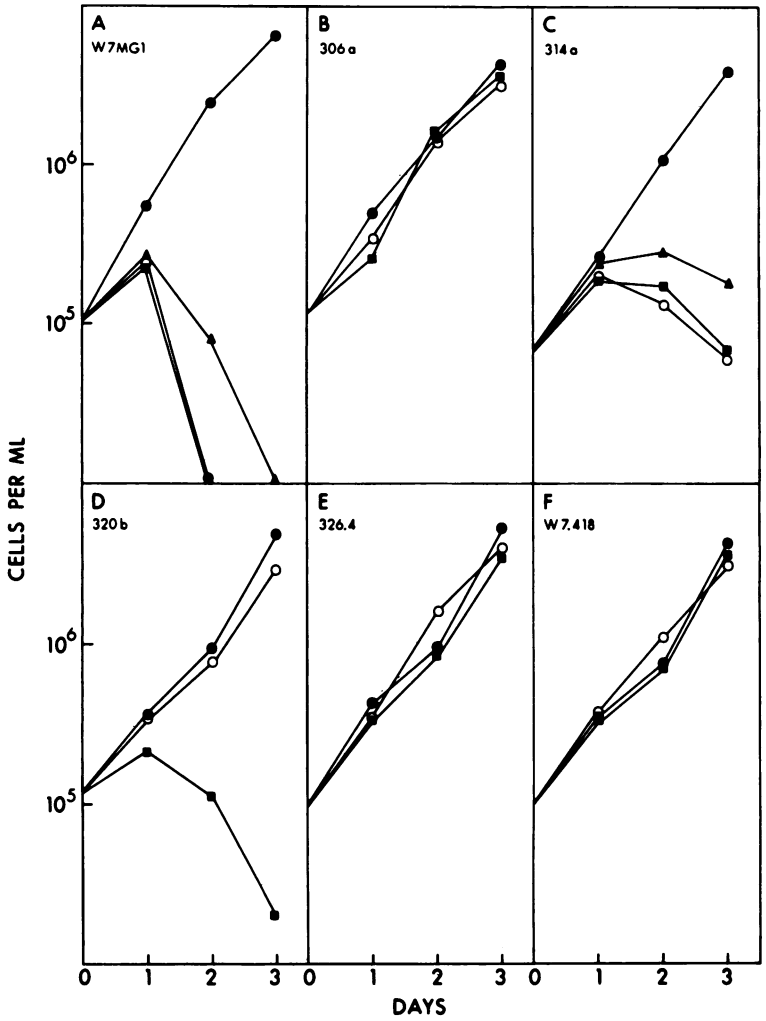

FIG. 1. Cell growth rates at various concentrations of dex. Concentrations of dex used were: ●, none; ▲, 10^{-7} M; ○, 10^{-6} M; ■, 10^{-5} M. Each panel represents a different strain (indicated).

15). Thus, we conclude that the loss of the cytolytic response in strains 306a and 314a was due to an insufficient number of GR. Cells of variant 314a clearly retained some GR function, since their growth was inhibited by high dex concentrations. However, for 306a cells it was not clear from the growth curves whether the residual glucocorticoid-binding activity represented functional GR. The presence of active, hormonally responsive MMTV genomes in the parental W7MG1 cell line allowed us to conduct a series of additional tests to assess the glucocorticoid responsiveness of the variants.

TABLE 1. GR gene products of dex-resistant variants

Cell line	Relative [^{3}H]dex binding ± SD	Relative amount of GR RNA[a] ± SD	Relative amount of GR protein (estimated)[b]
W7MG1	1	1 ± 0.4	1
W7M306a	0.21 ± 0.04	0.3 ± 0.2	0.2
W7M314a	0.27 ± 0.02	0.4 ± 0.1	0.2
W7M320b	0.04 ± 0.01	0.5 ± 0.1	1
ADR6.M189D	0.00 ± 0.00	0.1 ± 0.1	0

[a] Purified cytoplasmic RNA was assayed for GR mRNA content by slot-blot hybridization.

[b] Values were estimated by visual inspection of the immunoblot data in Fig. 2.

The relative levels of MMTV RNA were measured by cytoplasmic dot hybridization, and a ratio was calculated for hormone-treated versus control cells (Table 2). The relative levels of MMTV proteins on the surface of hormone-treated and control cells were measured by immunocytofluorometric analysis (Fig. 3) and by a panning assay (Table 2), in which binding of cells to petri dishes coated with a monoclonal antibody against the major MMTV glycoprotein (gp52) was measured. In W7MG1 cells the MMTV RNA level increased four- to fivefold in response to dex. This increased RNA level and the independent stimulation of the maturation of MMTV proteins (21, 22) were reflected in the increased level of MMTV proteins on the cell surface. In the cytofluorometric analysis of cells labeled indirectly with antiserum against total MMTV proteins (Fig. 3, bottom) or against gp52 (top), dex caused a substantial shift in the fluorescence distribution obtained for W7MG1 cells. The percentage of cells that bound to antibody-coated petri dishes increased from 2 to 85% in response to the hormone (Table 2).

For the two variants, hormonal stimulation of MMTV gene expression was also clearly observed, although the

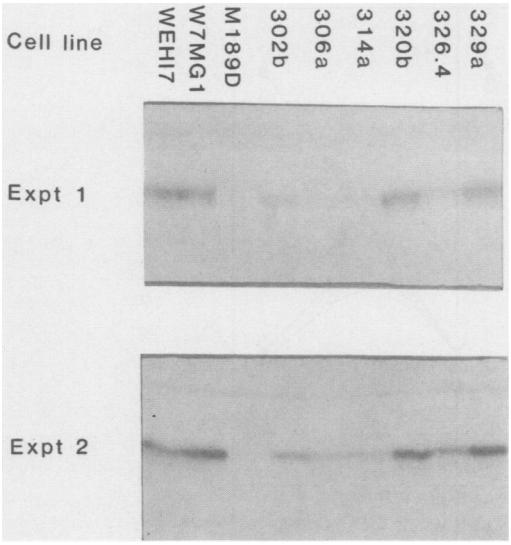

FIG. 2. Quantitation of GR by immunoblot. Results for two independently prepared extracts are shown for each cell line. The stained band migrated at approximately 94K. ADR6.M189D served as a negative control. Extracts from several previously characterized variants (21) were included for comparison. The hormone-binding activities of these additional variants, relative to W7MG1, were: 302b, 78%; 326.4, 53%; and 329a, 93% (22).

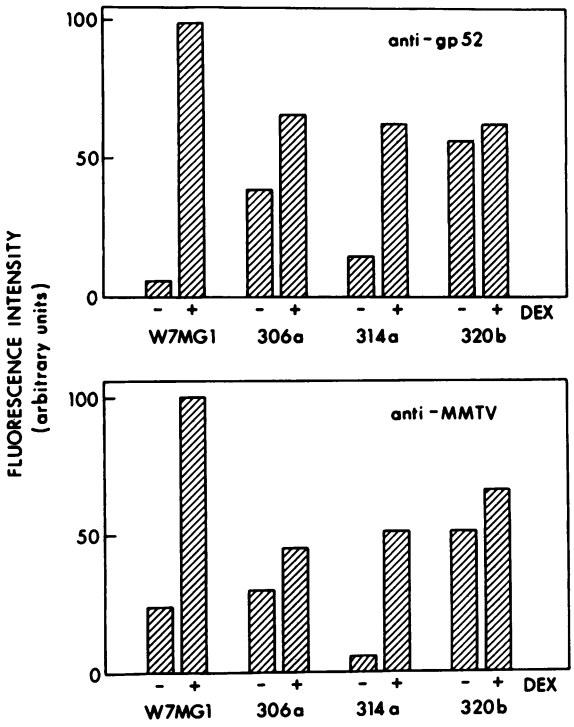

FIG. 3. Immunocytofluorometric analysis of MMTV proteins on the cell surface. Control cells or cells grown for 8 h with 10^{-6} M dex were stained with a primary rabbit antiserum against MMTV gp52 (top panel, 1:300 dilution) or against total MMTV proteins (bottom panel, 1:300 dilution) and then with a secondary fluorescein isothiocyanate-labeled goat antiserum against rabbit immunoglobulins (1:64 dilution). The fluorescence analysis was performed on the logarithmic scale of a fluorescence-activated cell sorter, and the data are presented in arbitrary units based on the mean fluorescence channel number for each stained cell population. Zero fluorescence in the graph represents the mean fluorescence for similarly stained WEHI7 cells, which contain no detectable MMTV RNA. The mean fluorescence for the dex-treated W7MG1 cells was designated as 100.

magnitude of the increase was substantially less than in the parental cells. Independent measurements were made on different days for each type of assay, and W7MG1 was used as a standard for comparison in each experiment. In variant 314a dex caused a three- to fourfold increase in the MMTV RNA level; in 306a an increase of only about 50% was observed (Table 2). Panning (Table 2) and cytofluorometric (Fig. 3) analyses of 314a indicated that the level of MMTV glycoprotein gp52 on the cell surface was similar to that for W7MG1 before dex and increased to an intermediate level compared with W7MG1 after hormone treatment. The same tests indicated that 306a had slightly higher basal levels of cell surface gp52 than the wild-type cells, and dex caused a small but reproducible increase.

In W7MG1 cells the MMTV glycoprotein precursor is produced at a low level even in the absence of glucocorticoid, but the production of the mature (proteolytically processed) forms of the MMTV glycoproteins is dependent on glucocorticoid action (21, 22). Pulse-chase analyses of the MMTV protein species produced before and after hormone treatment demonstrated that this hormonal stimulation of viral protein processing also occurred in the two variants, 306a and 314a (data not shown). Thus, four different assays for glucocorticoid responsiveness of MMTV gene expression were used to demonstrate that variants 306a and 314a contained functional GR.

TABLE 2. Glucocorticoid stimulation of MMTV RNA and cell surface protein levels in variants with reduced hormone-binding activity

Cell line	Ratio of MMTV RNA induction[a]	Mean % of cells bound by panning[b] ± SD	
		Without dex	With dex
W7MG1 (parent)	4.5 ± 0.7	2 ± 0	85 ± 3
W7M306a	1.4 ± 0.2	15 ± 3	26 ± 5
W7M314a	3.7 ± 1.0	2 ± 1	43 ± 5
W7M320b	1.5 ± 0.4	10 ± 3	20 ± 7

[a] The cytoplasmic dot hybridization method was used. The data are expressed as the ratio of the densitometric values for fixed cytosol preparations from hormone-treated versus control cells. The mean and standard deviation for two independent experiments are given.

[b] Binding of control or hormone-treated cells to dishes coated with monoclonal antibody against MMTV glycoprotein gp52 is expressed as the mean and standard deviation for two independent experiments, with duplicate determinations in each experiment.

A dex-resistant variant with a GR mutation that reduces hormone-binding affinity. When W7M320b cells were exposed to 10^{-6} M dex for 8 h, the level of MMTV RNA increased about 50% (Table 2), and the level of MMTV glycoprotein on the cell surface was also increased by a small but reproducible amount, as determined by panning (Table 2) and cytofluorometric analysis (Fig. 3). The reduced magnitude of the hormone response suggested reduced GR function.

Variant 320b bound only about 4% as much [^{3}H]dex as W7MG1 (Table 1), and similar results were obtained in the [^{3}H]dexamethasone 21-mesylate binding studies (not shown). However, RNA and protein-blotting experiments indicated that 320b cells contained near-normal levels of GR RNA and protein, respectively (Table 1 and Fig. 2). Since the hormone-binding studies used subsaturating concentra-

tions of the labeled steroid, one possible interpretation of this apparent paradox was that the GR in 320b cells had a reduced binding affinity for the hormone.

The K_d for binding of [^{3}H]dex to the wild-type GR is about 10^{-8} M (6, 20). Attempts to measure directly the K_d for hormone binding to the GR in 320b cells were unsuccessful. The background of nonspecific binding (not competable with excess unlabeled hormone) increased in proportion to the concentration of the labeled hormone, so that at [^{3}H]dex concentrations above 10^{-7} M, specific binding could not be detected above the background, even for W7MG1 cells. As an alternative to the hormone-binding assays, the glucocorticoid responses of the wild-type and variant cells were measured at different concentrations of hormone (i.e., dose-response relationships). W7MG1 cells were killed by dex concentrations of 10^{-8} M or higher (21) (Fig. 1). The increase in the level of MMTV gp52 on the surface of W7MG1 and 314a cells was half-maximal at 10^{-8} M dex and almost maximal at 10^{-7} M dex (Fig. 4). At 10^{-6} M dex, the response was maximum, and there was no further increase in fluorescence at 10^{-5} M dex.

The dose-response curves for 320b cells were quite different from those for W7MG1 and the other variants. The lowest concentration of dex that elicited an increase in the level of gp52 on 320b cells was 10^{-6} M (Fig. 4). The curve then rose sharply as the concentration increased to 10^{-5} M. Cells could not be tested at higher concentrations of steroid hormone because of rapid loss of viability. The curves for W7MG1 and 314a did not increase between 10^{-6} and 10^{-5} M dex, whereas the curve for 320b increased dramatically between these two concentrations. These three curves, including the important differences near 10^{-5} M dex, were reproduced almost exactly in an independent experiment. The dex concentration that would produce a half-maximal response in 320b cells was predicted to be above 10^{-6} M; the precise value could not be determined, since a saturating concentration of dex was not reached. These observations are consistent with the existence of a mutant GR in 320b cells with a reduced hormone-binding affinity.

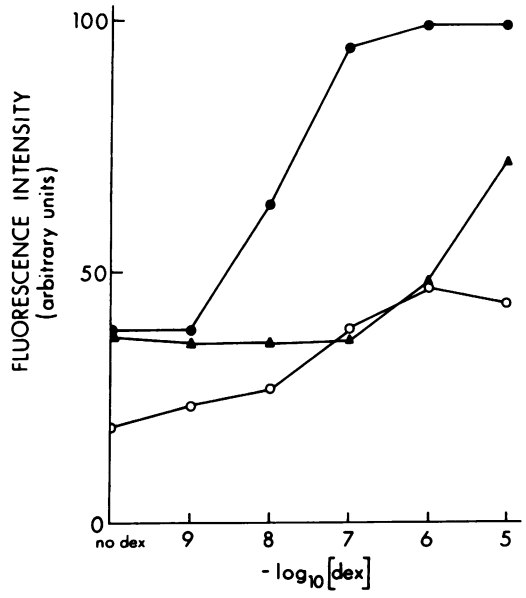

FIG. 4. Expression of MMTV proteins on the cell surface as a function of dex concentration. Cytofluorometric analyses of cell surface MMTV proteins were performed as described in the legend to Fig. 3 for W7MG1 (●), 314a (○), and 320b (▲) cells grown for 8 h in the indicated concentrations of dex. The primary antiserum used was against total MMTV proteins.

Similar dose-response curves were generated with the panning assay. The binding of 320b cells to antibody-coated petri dishes was unaffected by 10^{-7} M dex and then increased sharply between 10^{-6} M and 10^{-5} M dex, whereas the binding of W7MG1, 306a, and 314a had already reached maximum values at 10^{-6} M dex and did not increase further at 10^{-5} M dex (data not shown).

The results from growth curves conducted in different dex concentrations were also consistent with the conclusion that 320b cells contained GR with a reduced binding affinity for dex. Variant 320b grew normally in dex concentrations as high as 10^{-6} M but was killed by 10^{-5} M dex (Fig. 1D). In contrast, two other dex-resistant variants (W7M326.4 and W7.418) that lacked functional GR grew normally in 10^{-5} M dex (Fig. 1E and F). Thus, the cytolytic response to 10^{-5} M dex required functional GR, because the two receptor-deficient lines were not affected by that concentration of dex.

Two other steroids, progesterone and estradiol, at concentrations as high as 10^{-5} M failed to elicit increases in MMTV proteins on the surface of W7MG1 cells (by cytofluorometric analysis). Furthermore, at 10^{-6} M these two steroids had no effect on the growth of W7MG1 or 320b cells, and at 10^{-5} M they caused only a slight reduction in growth rate (data not shown). Thus, the response of 320b cells was specific for glucocorticoids and GR.

A WEHI7 variant with reduced membrane permeability for dex has been reported (16). In that study, whole-cell binding of labeled dex was lower for the variant than for the wild-type cells, but cell extracts from the two cell lines bound equal amounts of the hormone. However, in extracts from 320b cells, there was no detectable binding above the background level of 2,900 ± 300 cpm (mean ± standard deviation) at 5×10^{-8} M [^{3}H]dex, whereas extracts from W7MG1 cells bound 3,300 ± 80 cpm above a similar background. Thus, the GR protein from 320b cells, although present in approximately normal amounts, had a reduced ability to bind hormone. We propose that the GR in 320b cells has a structural mutation that affects hormone binding.

DISCUSSION

Hormone-resistant variants with low GR levels. In three previous studies in which glucocorticoid-resistant variants of T-lymphosarcoma cell lines were selected (11, 20, 23, 30), the majority of the variants (60 to 80%) had no detectable glucocorticoid-binding activity (i.e., less than 2 to 3% of the wild-type level). The remainder of the variants exhibited glucocorticoid-binding activities ranging from about 5 to 50% of the wild-type level. Some of these variants (nt^- and nt^i) clearly had altered forms of the GR molecule which accounted for their lack of responsiveness (reviewed in reference 15). However, many of the steroid-resistant variants that retained some hormone-binding activity appeared to have GR with no obvious physical abnormalities. The hormone resistance in these variants was presumably due to lack of sufficient numbers of functional GR; however, without a functional test for the remaining receptors, it was not possible to determine whether the residual glucocorticoid-binding activity in these cells represented functional or nonfunctional GR.

In the studies reported here, the MMTV marker provided a convenient and much more sensitive assay for GR func-

tion. Thus, we were able to demonstrate for the first time the existence of a variant (306a) that grew normally in high glucocorticoid concentrations but contained functional GR. Since previous studies have established that GR levels in excess of 25% of wild-type levels are required to support the growth inhibition and cytolytic responses, it is likely that the resistance of this variant to cytolysis was due largely to the reduced GR level. The fact that the GR mRNA level, protein level, and hormone-binding activity in this variant were all proportionately lower than in wild-type cells indicates that the lesion affected GR gene transcription, RNA processing, or mRNA stability.

Although 306a and 314a had similar GR levels, the magnitude of the hormone responses of 314a was substantially greater than that of 306a. Two possible explanations for the relatively low hormone responsiveness of 306a cells are that only a fraction of the GR in 306a cells may be functional or that 306a cells may contain a secondary defect that compromises hormone responsiveness. The reason for the elevated basal levels of MMTV gene expression in some of the variants is unclear.

Another implication of this variant phenotype is that fewer GR are required to carry out the hormonal stimulation of MMTV transcription and protein processing than are required to carry out the growth inhibition and cytolysis responses. Possible explanations for this difference include chromosomal target sites with different affinities for the receptor-hormone complex or a requirement for multiple receptor-binding interactions to trigger the cytolytic response. It is also noteworthy that the magnitude of the MMTV responses appeared to correlate roughly with the GR level; this observation, along with the threshhold requirement for the cytolytic response, suggests that GR level is a limiting factor in the general glucocorticoid responsiveness of these cells. A similar conclusion was recently reached by Vanderbilt et al. (28) from examination of rat hepatoma cell lines with varying GR contents.

Mutant GR with reduced hormone-binding affinity. RNA and protein-blotting experiments indicated that variant 320b had normal or near-normal levels of GR gene expression. However, this variant bound [^{3}H]dex very poorly at concentrations as high as 5×10^{-8} M and responded very poorly to concentrations of dex (10^{-6} M) that elicited a maximum response from wild-type cells and other variants with reduced levels of normal GR. Higher dex concentrations elicited a substantial increase in the response of 320b cells. The difference in the dose-response curves between 320b and the other cell lines strongly suggested that 320b cells had GR that were functional but had a greatly reduced hormone-binding affinity. Since the GR in 320b appeared to be normal in size, the most likely explanation for this phenotype is an amino acid substitution in the hormone-binding domain of the GR. The absence of GR with wild-type functional or hormone-binding characteristics in these cells suggests that one GR gene is producing no protein product or one that is totally nonfunctional and the other GR gene is producing the partly functional GR with the reduced hormone-binding affinity.

At this point we have not demonstrated directly that the responses of 320b cells to 10^{-5} M dex were mediated by the GR. Theoretically, at such high, nonphysiological concentrations, dex might interact with the progesterone receptor (if present in these cells) or might act through some other nonspecific pathway. However, observations of two independent glucocorticoid responses provided strong indirect evidence that these responses were mediated by a mutant GR in 320b cells. First, variant 320b was killed by 10^{-5} M dex, but two other dex-resistant lines, 326.4 and W7.418, were not; the latter two lines are from the WEHI7 lineage and lack functional GR. Thus, the cytolytic response of 320b cells at 10^{-5} M dex but not at lower concentrations was peculiar to this variant and was not a general characteristic of the WEHI7 lineage. Moreover, the cytolytic response to 10^{-5} M dex required a functional GR. Second, a similar argument applies to the dose-response curves for the expression of MMTV proteins on the cell surface. By comparing the response profiles of W7MG1, 314a, and 320b, it is clear that the increased response of 320b cells between 10^{-6} and 10^{-5} M dex was peculiar to 320b cells and not a general characteristic of W7MG1 and its derivatives. Finally, neither the MMTV response nor the cytolytic response was elicited by progesterone or estradiol, eliminating the possibility that dex was acting through these other steroid receptors.

The glucocorticoid-binding domain of the GR has been mapped to a region of approximately 250 amino acids at the C terminus of the protein by DNA deletion-transfection mapping analyses (8, 13). In addition, the precise site of covalent dexamethasone 21-mesylate binding has been assigned to Cys-656 (25). Several amino acid substitutions that affect hormone binding have also been mapped: alteration of amino acid 546 eliminated hormone binding, and another change at residue 770 reduced hormone-binding affinity by three- to fourfold (4). Clearly, mutations in a broad region of the protein can affect hormone binding, and more point mutations will be required to map the critical amino acid residues for this function. Mapping of this broad region will require mutants obtained by classic somatic-cell genetics as well as mutants generated in vitro. Variant 320b is the first reported mutant that has a functional steroid receptor with a severely reduced binding affinity for the hormone.

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