Down-Regulation of Glucocorticoid Receptors in Mouse Lymphoma Cell Variants

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The mouse thymoma-derived cell line W7 is sensitive to the cytolytic action of glucocorticoids. We have isolated a novel class of cell variant that apparently overcomes its inherent sensitivity to glucocorticoids by reversibly down-regulating the level of glucocorticoid receptors. This phenotype is stable during subcloning in the presence and in the absence of glucocorticoids and is dominant in somatic cell hybrids with wild-type cells. Fusion of this variant with wild-type cells produces hybrids that down-regulate and are less sensitive to glucocorticoids than hybrids of receptor-negative and wild-type cells. This is the first demonstration of a phenotypic change which correlates with down-regulation of the glucocorticoid receptor.

Exposure of mammalian cells to a hormone frequently results in a reversible alteration in the cellular concentration of receptors for that hormone. Estrogen causes an elevation in the level of its receptor in tubular gland cells of the chick oviduct (12). Thyroid hormones, in contrast, down-regulate their nuclear-binding proteins (17). Hormones that interact with receptors on the cell surface also frequently cause down-regulation of their receptors; examples are insulin (15) and epidermal growth factor (3). Down-regulation of glucocorticoid receptors has been reported in HeLa cells (4) and in AtT-20 pituitary tumor cells (19); however, no corresponding change in a biological response was observed.

Many T-lymphocytes are killed by physiological concentrations of glucocorticoids (5), and some thymoma-derived cell lines retain their cytolytic response to glucocorticoids (8, 9). Genetic studies of the cytotoxic response have produced a large number of hormone-resistant variants with defective glucocorticoid receptor proteins (reviewed in reference 10). We are attempting to isolate new types of glucocorticoid response variants from the WEHI7 mouse lymphoma cell line to identify other genes involved in glucocorticoid response pathways. We describe here lymphoma cell variants that become resistant to the cytolytic and growth inhibitory effects of glucocorticoids after down-regulating the level of their glucocorticoid receptor proteins in response to the hormone.

MATERIALS AND METHODS

Materials. Dexamethasone (Dex) and polyethylene glycol 1000 were purchased from Sigma Chemical Co.; [3H]Dex was from New England Nuclear Corp.; [3H]uridine was from ICN Pharmaceuticals Inc.; powdered tissue cultured media was from Flow Laboratories, Inc.; and heat-inactivated horse serum was from KC Biologicals.

Cell lines and cell culture techniques. W7.2 is a subclone of mouse T-lymphosarcoma cell line WEHI7 (9). W7MG1 is a Dex-sensitive, mouse mammary tumor virus-infected, hypoxanthine-guanine phosphoribosyltransferase-negative (HGPRT−) subclone of W7.2 (6). W7TB and W7:418 are thymidine kinase-negative subclones of WEHI7; W7TB is fully Dex sensitive (receptor positive), and W7:418 is fully Dex resistant and has no measurable glucocorticoid-binding activity (1). W7 MS1 is a partially Dex-resistant variant of WEHI7 (1). All cultures were grown in suspension in Dulbecco modified essential medium supplemented with 10% heat-inactivated horse serum. For growth curves, cells were counted with a hemocytometer and an inverted phase-contrast microscope. Live cells are large, turgid, translucent spheres that are easily distinguished from dead cells which are shrunken, irregular in shape, and refractile. Viable cell counts obtained by using these visual criteria were essentially identical to those obtained by observing trypan blue exclusion.

Somatic cell hybrids were formed by the polyethylene glycol fusion method (7). Cells (5 × 106) of each clone were mixed, washed with 5 ml of growth medium, and suspended in 0.5 ml of 50% (wt/vol) polyethylene glycol 1000 at 25°C. After 3 min, cells were washed extensively with medium and then suspended in medium containing 10% horse serum. Hybrids were selected in multwell titer plates with HAT medium (11). All hybrid clones were verified by determining approximate chromosome counts for several randomly chosen fixed cells from the cloned population. The average chromosome count for each hybrid clone was between 70 and 75. For W7.2 cells counted by the same method, the average count was 36.

Quantitation of glucocorticoid binding to whole cells. Glucocorticoid binding and nuclear translocation were measured with a whole-cell [3H]Dex-binding assay (14). For each clone, the number of receptors per cell and the KD were determined (Table 1) from a Scatchard plot of these data by the Rosenthal correction method (16). The standard error is given where appropriate.

In subsequent experiments, the number of receptors per cell was determined by measuring [3H]Dex binding at a single nonsaturating concentration of the ligand (approximately equal to the KD). The results presented are the average of three determinations. The absolute counts bound per cell varied less than 10% for a given experiment, but the average counts bound varied considerably from day to day. Therefore, W7.2 cells were used as a quantitative standard in each experiment; the receptor numbers in Table 2 and Fig. 2 were calculated relative to W7.2 (33,000 receptors per cell, as determined in Table 1). These determinations provide a relative measure of receptor number that is reproducible from experiment to experiment; however, the Scatchard analyses in Table 1 may provide a more accurate absolute value. For clone ADR6, the data from the two methods were in good agreement; however, for clone ADR38, the number of receptors determined by the two methods, although

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reproducible in each case, did not agree (compare Tables 1 and 2).

For comparison of \(^{3}H\)
Dex binding to cells before or after long-term Dex treatments, two steps were added to ensure that unlabeled Dex (present in some cultures but not in others) would not cause artifactual differences in the \(^{3}H\)
Dex-binding data. For cultures growing without Dex, 1 
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M Dex was added to the growing culture for 1 h. Next, all cultures were washed three times by centrifugation in
Dex-free medium and then grown for 1 h at 37°C without Dex before the \(^{3}H\)
Dex-binding assay was performed. In a control experiment, cells grown in 1 
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M unlabeled Dex for 1 h and then washed by the above procedure bound the same amount of \(^{3}H\)
Dex as cells that were not previously exposed to unlabeled Dex.

RESULTS

Some Dex-resistant clones exhibit a growth lag when first exposed to the hormone. W7.2 cells were mutagenized by incubation for 16 h at 37°C in growth medium containing 250 
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g of ethyl methanesulfonate per ml. The surviving cells (15%) were grown for 3 days and then plated at high density (10 \(^{6}\) cells) on 60-mm agar plates (13) containing 1 
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M Dex. Cloning in Dex-containing medium provides a rigorous selection for resistance to the cytolytic effect of the hormone. Resistant colonies appeared at a frequency of 4 \(\times\) 10
\(^{-7}\) and were propagated in Dex-free medium for several weeks. To identify variants with near-normal levels of active receptor, the receptor content and the nuclear translocation of hormone-receptor complexes were measured for each clone by a whole-cell \(^{3}H\)Dex-binding assay (see above). Out of 60 clones examined, 10 (labeled ADR) had at least 30% of the wild-type level of receptor and apparently normal percentages (60 to 70%) of nuclear translocation of the hormone-receptor complex. Growth rate analyses of each clone in the presence or absence of 1 
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M Dex revealed that 3 of the 10 ADR clones (ADR6, ADR9, and ADR19) were temporarily growth inhibited by 1 
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M Dex after long-term growth in the absence of the hormone (Fig. 1A). After addition of Dex to the medium, these cells grew normally for 1 day and then exhibited a growth lag for 1 to 2 days before resuming growth at a rate slightly slower than their pre-Dex rate. During the lag phase, numerous dead cells appeared. Wild-type W7 cells, in contrast, ceased growth within 12 h of Dex addition and underwent cytolsis within 24 to 36 h, leading to a precipitous drop in the growth profile (Fig. 1B). The growth profile of fully Dex-resistant clones, such as ADR38, was identical in the presence or absence of Dex, with no lag (Fig. 1C).

ADR6 cells were grown continuously in 1 
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M Dex for 2 months and then changed into Dex-free medium for 3 days. When Dex was returned to the medium at this point, logarithmic growth continued without any lag. However, if the Dex-conditioned cells were grown without Dex for several weeks before returning Dex to the medium, the growth lag phenotype returned.

The lag phenotype of the three ADR clones persisted after two rounds of subcloning, indicating that the lag was not due to a mixed population of sensitive and resistant cells in the original ADR isolates. Subclones grown in Dex for 60 generations and then recloned in the absence of Dex (a process requiring several weeks) still exhibited the lag phenotype when Dex was returned to the medium.

Wild-type W7 and fully Dex-resistant ADR38 cells were mixed together and grown in 1 
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M Dex (Fig. 1D). The growth profile of this mixed population in Dex did not resemble that of ADR6 in Dex (compare Fig. 1A and 1D). First, the number of live cells in the mixed population decreased on the first day, whereas the number of ADR6 cells increased for 1 day after Dex addition. Second, the mixed population resumed logarithmic growth after 1.5 days, whereas ADR6 lagged until day 3. Various ratios of sensitive and resistant cells were grown together, and in no case was the growth profile of the mixture similar to that of ADR6. This result further supports the conclusion that the lag phenotype of ADR6 is not due to a mixture of resistant and sensitive cells.

Down-regulation of glucocorticoid receptors in the growth-lag variants. Glucocorticoid receptors from the growth-lag clones (ADR6, ADR9, and ADR19) and wild-type cells have the same binding affinity for Dex, within experimental error (Table 1). However, these three variants have only 30 to 60% as many receptors as wild-type cells.

A 50% reduction in wild-type receptor levels should not, of itself, prevent cytolysis from occurring in W7 cells exposed

| TABLE 1. Glucocorticoid receptors of wild-type W7.2 and Dex-resistant ADR clones |
|----------------|---------|----------------|
| Cell line      | Receptors per cell* | K_d*          |
|----------------|---------|----------------|
| W7.2           | 33,000  | 12             |
| ADR6           | 15,600 ± 950 | 12 ± 2        |
| ADR9           | 9,700 ± 300  | 10 ± 3       |
| ADR19          | 19,100 ± 2000 | 13 ± 1       |
| ADR38          | 7,400 ± 800   | 17 ± 3       |

* The number of glucocorticoid receptors per cell and the K_d (in nanomoles per liter) for Dex were determined from a Scatchard analysis as described in the text. The standard error is given where appropriate.
Dex level after 3 weeks; however, in a separate experiment, the receptor level of ADR6 cells rose fourfold after 2 weeks in the absence of Dex but only reached about 40% of the original level (Table 2). In semilogarithmic plots of the receptor down-regulation data, the changes in receptor levels do not conform to simple first-order kinetics.

The large change in receptor content per cell within the first day after hormone addition or withdrawal cannot be due to selective survival of a subpopulation of cells for the following reasons. First, no cell death is observed after the withdrawal of Dex or within the first 24 h after the addition of Dex to nonadapted cells. Dead cells do finally appear in the Dex-treated cultures, but only after 2 days, by which time the rapid initial decrease in receptor number is complete. Second, the withdrawal of Dex from Dex-conditioned cells should not create any strong selective pressure, but nevertheless it results in a dramatic, rapid increase of receptor levels. These results suggest that ADR6 overcomes Dex sensitivity by a reversible down-regulation of the level of glucocorticoid receptors.

Two other ADR clones were examined for down-regulation of glucocorticoid receptors in response to Dex. ADR33 and ADR38 are both fully Dex resistant and exhibit no growth lag when exposed to 1 \( \mu M \) Dex. ADR38 exhibits a reversible down-regulation of its glucocorticoid receptors (about 50%) when grown in Dex, but ADR33 retains approximately the same number of receptors per cell when grown with or without Dex (Table 2). The lack of change in ADR33 receptor levels is a further indication that the dramatic down-regulation in ADR6 is not an artifact.

ADR clones 6, 9, and 19 were derived from a single mutagenized population of W7.2 cells. Therefore, it is theoretically possible that they are sibling clones. However, since the level of glucocorticoid receptor is quite different in each clone, it seems unlikely that they are siblings. ADR38 is derived from an independently mutagenized population of W7.2 cells.

The rapid cytolytic response of wild-type W7 cells precludes long-term measurements on receptor down-regulation. We exposed W7.2 and MS1 to 1 \( \mu M \) Dex and measured

![FIG. 1. Growth curves of sensitive and resistant WEHI7 clones in the presence and absence of Dex. Growth analyses were performed as described in the text. Dex-resistant clones ADR6 and ADR38 are also described in the text. Symbols: (A, B, and C) ○, no Dex; ●, 1 \( \mu M \) Dex; □, 1 \( \mu M \) Dex; ▲, ADR38 in 1 \( \mu M \) Dex; ■, a mixture of W7 and ADR38 grown in 1 \( \mu M \) Dex. At time zero, the cell density for each clone in the mixed population was the same as in the corresponding culture.](image1)

![FIG. 2. Down-regulation of glucocorticoid receptor levels in the Dex-resistant variant ADR6. (A) ADR6 was grown without Dex for several weeks, and 1 \( \mu M \) Dex was added at time zero. Glucocorticoid receptors were determined relative to W7.2 as described in the text. (B) ADR6 was grown in 1 \( \mu M \) Dex for several weeks, and then the cells were transferred to Dex-free medium at time zero. Receptor levels were determined as above.](image2)
receptor levels at the time point at which dead cells normally start to appear (~15 h for W7.2 and ~36 h for MS1). W7.2 showed a 30% decrease in receptors after 15 h of Dex treatment, whereas MS1 showed a 20% decrease at 15 h in one experiment and no decrease in another experiment. It must be stressed here that a clear interpretation of these results with Dex-sensitive cells is not possible due to the imminent cytolysis of these cells.

As an alternative, we examined HTC, a line of rat hepatoma cells with several well-documented glucocorticoid responses (20). The growth and survival of HTC cells are not adversely affected by glucocorticoids. HTC cells grown for 1 day in 1 μM Dex have only 30 to 40% as many receptors as HTC cells grown without Dex or exposed to Dex for only 1 h (Table 2).

**Somatic cell hybridization studies with ADR6.** Cell hybrids were constructed between ADR6 and receptor-positive or receptor-negative WEHI7 clones. A HGPRT- derivative of ADR6 was fused with the fully Dex-sensitive (receptor-positive) clone W7TB or the fully Dex-resistant (receptor-negative) clone W7.418. W7TB and W7.418 are thymidine kinase-negative derivatives of WEHI7 and should be isogenic except for the glucocorticoid receptor genes. The Dex-sensitivity of these hybrids was compared with that of two control hybrids: W7.418 (receptor negative) × W7MG1 (receptor positive) and W7TB (receptor positive) × W7MG1 (receptor positive). W7MG1 is a HGPRT+ derivative of mouse mammary tumor virus-infected W7.2 cells; W7MG1 is fully Dex-sensitive and has a wild-type level of glucocorticoid receptors. The parental lines and three to five HAT-resistant clones from each fusion were analyzed for growth in the presence or absence of Dex.

Figure 3 shows the growth curves of representative hybrid clones in the presence and absence of Dex. The fusion of two Dex-sensitive lines (W7TB × W7MG1) yields fully Dex-sensitive hybrid clones that die within 2 days of exposure to 1 μM Dex (Fig. 3a). The hybrids of a Dex-resistant clone with a Dex-sensitive clone (W7.418 × W7MG1) are also killed by exposure to 1 μM Dex (Fig. 3b) but survive lower concentrations better than the W7TB × W7MG1 hybrids (data not shown). In contrast, the W7TB × ADR6 hybrids were partially resistant even to 1 μM Dex (Fig. 3d); after 2 weeks in 1 μM Dex, viable cells were still present in the population, although no net increase in cell number had occurred. Transfer of these cells to Dex-free medium allowed resumption of normal growth. However, a fully resistant population was not obtained, even after 3 weeks in 1 μM Dex. In 0.1 μM Dex, these hybrids exhibited a growth lag for several days and then resumed logarithmic growth (data not shown). Thus, the Dex sensitivity of these W7TB × ADR6 hybrid clones is intermediate compared with the two parent clones; the Dex-induced growth lag peculiar to the ADR6 parent is also observed in the hybrid but only at a lower Dex concentration (0.1 μM). The W7.418 (receptor negative) × ADR6 hybrid clones were fully resistant to 1 μM dex with no Dex-induced lag in their growth profiles (Fig. 3c).

[HI]Dex-binding experiments were performed on the W7TB × ADR6 and the W7.418 × ADR6 hybrid clones before and after a long-term (1 to 3 weeks) exposure to Dex (Table 2). Dex causes down-regulation of receptors in these hybrids, as in the ADR6 parent clone. The receptor down-regulation in the hybrid clones was partially reversible.

**DISCUSSION**

In diploid and tetraploid lymphoma lines derived from the WEHI7 cell line, the degree of sensitivity to glucocorticoids correlates well with the wild-type receptor content of the cells (2). Our results generally support these findings and suggest that for a given concentration of Dex, there is a threshold level of receptor required for growth inhibition or cytosis. In addition, our results suggest that receptor down-regulation can influence the sensitivity of these cells to glucocorticoids.

Bourgeois and Newby (1) and Danielsen (unpublished data) have isolated Dex-sensitive WEHI7 clones with approximately half the wild-type receptor level. Thus, a 50% reduction in wild-type receptor levels does not normally prevent cytosis from occurring when WEHI7 cells are exposed to 1 μM Dex. However, several of the ADR clones can grow on 1 μM Dex, even though they have 50% or more of the wild-type level of receptors (e.g., ADR6 measured before Dex treatment). In fact, these variant lines were originally isolated by cloning in the presence of 1 μM Dex. The receptors of these clones have normal binding affinity for Dex and normal levels of nuclear transfer of the Dex-receptor complex. The apparent inconsistency of the receptor level and the level of Dex sensitivity in the ADR growth-lag clones can be explained by our observation that these cells down-regulate their glucocorticoid receptor level in response to the hormone. It follows that the ability of these cells to survive in Dex depends on their ability to down-regulate receptors fast enough to avoid the cytolytic response. It appears that the surviving ADR6 cells narrowly avoid cytosis, since numerous dead cells appear in the culture during the lag. Higher receptor levels are apparently required for cytosis than for down-regulation (reported here) or for induction of mouse mammary tumor virus gene expression (unpublished data).

ADR6 cells have a high enough basal level of receptors to experience a growth lag before receptor down-regulation takes effect. However, in the W7.418 × ADR6 hybrids, the
relative dosage of active receptors is reduced by a factor of 2, and this apparently reduces the receptor level below the critical point necessary for an observable Dex-induced growth lag. W7TB × ADR6 hybrids, which exhibit a flat growth profile in 1 μM Dex (Fig. 3d), apparently have enough receptors to be growth-inhibited by 1 μM Dex but not enough receptors to cause efficient killing of the whole population. When these cells are preconditioned by growth in 0.1 μM Dex, they can subsequently grow in 1 μM Dex (data not shown). It is particularly interesting that the W7TB × ADR6 hybrids are less sensitive to Dex than the W7.418 × W7MG1 hybrids, even though the former hybrids should have more receptors per cell than the latter (cf. reference 10). This lower-than-expected Dex sensitivity of the W7TB × ADR6 hybrids and the growth lag exhibited in 0.1 μM Dex before achieving logarithmic growth are consistent with our observation that down-regulation of receptors is occurring in these hybrids. The lag in down-regulation of the hormone resistance of ADR6 appears to be dominant; on the contrary, hormone resistance due to nonfunctional receptors (W7.418) is recessive in hybrids.

It is an unfortunate characteristic of our W7 cell system that down-regulation cannot be studied in the wild-type cells. Even when receptor measurements are performed after relatively brief periods of Dex treatment, before any cytolyis is observed (15 h for W7.2 and 15 to 36 h for MS1), the fact that the cells are rapidly approaching cytolyis makes any interpretation of the results for Dex-sensitive cells extremely tenuous. Meaningful results can only be obtained from healthy, growing cells. The changes in receptor levels at earlier times (e.g., after 8 to 10 h of Dex treatment) would be too small to have any real relevance. Thus, a direct comparison of receptor down-regulation in variant and wild-type cells is not possible.

Although we cannot rule out the possibility that variants like ADR6 are deficient in a thus-far undefined function required for the cytolyis response, the results summarized below strongly suggest a different conclusion: namely, (i) that the dramatic down-regulation observed with ADR6 is not characteristic of wild-type WEHI7 cells but instead is a novel phenotype of the ADR variants and (ii) that the down-regulation of glucocorticoid receptors is responsible for the unusual type of Dex resistance of ADR6 and its somatic cell hybrids. In support of this conclusion, first, many W7 variants, such as MS1, with glucocorticoid receptor levels comparable to or lower than our ADR variants (grown without Dex) are killed by 1 μM Dex or even 0.1 μM Dex. Second, the Dex-induced growth lag of ADR6 coincides with the down-regulation of receptors; and when Dex is withdrawn from Dex-conditioned ADR6 cells, the slow reappearance of the growth lag is coincident with the slow restoration of receptors to their original pre-Dex level. Third, ADR6 × wild-type hybrids are more Dex resistant than receptor-negative × wild-type hybrids, despite the higher initial receptor levels of the former hybrids.

Although down-regulation of glucocorticoid receptors has been previously reported in HeLa cells (4), AT-T20 pituitary tumor cells (19), and human peripheral lymphocyte populations (18), our data demonstrate for the first time the loss of a biological response (hormone-mediated cytolyis) associated with the down-regulation of glucocorticoid receptor levels in a clonally derived cell population. Because of their Dex resistance, the ADR variants should facilitate the study of down-regulation of glucocorticoid receptors in W7 cells.

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