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Glucocorticoid-Induced Lymphocytolysis: State of the Genetic Analysis

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The glucocorticoid-induced lysis of lymphoid cell lines offers a genetic approach to steroid hormone action because unresponsive variants can easily be selected as resistant to this lytic effect. The present state of analysis of lymphocytolysis in two murine cell lines, the S49 T-lymphoma and the W7 thymoma, is reviewed. All glucocorticoid-resistant variants isolated so far result from various defects in the glucocorticoid receptor. The absence of variants blocked at another step of the lytic mechanism is discussed. The observed hemizygosity of the glucocorticoid receptor locus in the S49 line and the instability of cell hybrids illustrate some of the potential problems encountered in somatic cell genetics.

Key words: glucocorticoids, glucocorticoid receptor, lymphocytolysis, T-lymphoma, thymoma, cell variants, cell hybrids

All steroid hormones appear to act by a general mechanism involving a steroid-specific cytoplasmic receptor which, upon binding of the steroid, undergoes a conformation change called "activation." As the result of this activation step, the receptor-steroid complex acquires DNA binding capacity and enters the nucleus, where its interaction with a "nuclear acceptor" affects gene expression. Although a wealth of data support this model [reviewed in references1–3], the nature of the nuclear acceptor is still unknown, and the steps beyond nuclear transfer are entirely obscure. The current view is that the nuclear acceptor is chromatin and that steroid receptors act at the level of transcription.

An attractive approach to elucidate the mode of action of steroid hormones is the use of cloned cell lines responsive to a steroid in vitro, and the isolation and characterization of unresponsive variants. This has been possible in the case of glucocorticoid receptors because murine lymphoid cell lines are available that undergo lysis in the presence of glucocorticoid hormones. This response allows the isolation of rare variants resistant to the killing effect. The ease in selecting for glucocorticoid-resistant lymphoid cell variants has made this system ideal for somatic cell genetic studies of steroid hormone action. This paper will briefly review the present state of analysis of that system, pointing out some of its limitations as well as its advantages. These studies also illustrate some of the general problems that can be encountered in using cell lines established in culture and in analyzing cell hybrids.

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HEMIZYGOSITY OF THE GLUCOCORTICOID RECEPTOR GENE

The mouse T-lymphoma line, S49 [4], has been extensively used for such studies. However, some properties of the S49 line raised questions about the validity of using that line for a genetic analysis of the mechanism of lymphocytolysis. In particular, the frequency of appearance of glycocorticoid-resistant S49 variants, of the order of 10^{-6} to 10^{-5} is surprisingly high for a genetic event in a pseudo-diploid somatic cell [5–8]. High mutation rates have been observed at a variety of loci, in particular in Chinese hamster ovary lines where they have been interpreted as resulting from functional hemizygosity [9]. In the case of glucocorticoid-resistant variants of the S49 line, however, this explanation is insufficient, because this high frequency appeared to be independent of cell ploidy, in that it was observed in a pseudo-tetraploid S49 line as well as in pseudo-diploids [5–7]. This behavior suggested the possibility that the appearance of resistance involved a stable phenotypic variation in the expression of the genome, rather than a change in the primary structure of DNA – ie, an "epigenetic" rather than a genetic event.

Because of this and other problems encountered with the S49 line, we have turned to another glucocorticoid-sensitive lymphoid line, the murine thymoma WEHI-7 (W7) [10]. Our interest in the W7 line was prompted by the observation by A. Harris [personal communication] that no dexamethasone-resistant variant could ever be spontaneously derived from this line, in contrast to the high frequency of such variants mentioned earlier for the S49 line. In fact, no resistant variant was found when populations of approximately 8×10^9 W7 cells were exposed to 10^{-6}M dexamethasone, confirming that the frequency of this event is very low, $< 1.2 \times 10^{-10}$. This frequency can be increased to values of 10^{-7} to 10^{-6} by mutagenic treatment with N-methyl-N′-nitro-N′nitrosoguanidine [8] or other mutagens [11], as was shown to be the case for the S49 line [6].

This striking difference between the S49 and W7 lines encouraged us to compare other relevant properties of these two lymphoid lines, in particular their glucocorticoid receptor content and their level of sensitivity to dexamethasone [8]. The results of this comparison and our interpretation of the data are summarized in Table I.

In a whole cell binding assay [12], the W7 line was found to contain 30,000 dexamethasone binding sites per cell, while the S49 line contained only half that amount, or 15,000 dexamethasone binding sites per cell [8]. The glucocorticoid receptors of both lines appear identical in that they have the same affinity for dexamethasone, Kd = $1.3 \pm 0.3 \times 10^{-8}$M [8, 12], and that the same fraction of receptor–steroid complexes is transferred to the nucleus, namely 70% in the conditions of our nuclear transfer assay [12]. Moreover, quantitative tests for sensitivity to killing by dexamethasone revealed that, while both lines are sensitive to dexamethasone concentrations of 10^{-6} to 10^{-5}M, the S49 line is resistant to low concentrations of 10^{-9} to 10^{-8}M dexamethasone, which are sufficient, however, to kill the W7 line.

These observations led us to formulate the following working hypothesis: while two copies of a gene, r^+, coding for the glucocorticoid receptor would be present in the W7 line (r^+/r^+), as expected for a psuedo-diploid somatic cell, the S49 line would be functionally hemizygous for that locus (r^+/r^-). This interpretation of our results would also explain the observation that glucocorticoid-resistant variants arise at much lower frequency from W7 than from S49. Indeed, in the latter case, a single genetic event is required to inactivate the r^+ allele, while two events would be necessary to inactivate both copies of the r^+ gene in the W7 line.

TABLE I. Two-Step Model of Acquisition of Resistance*

Receptor alleles	$r^+/^+$	$\xrightarrow{\sim 10^{-6}}$ r^+/r^-	$\xrightarrow{\sim 10^{-6}}$ r^-/r^-
Cell line	W7	S49	Dex^R
Frequency of Dex^R	$\sim 10^{-12}$	$\sim 10^{-6}$	1
Dexamethasone binding sites per cell	30,000	15,000	0 or altered
Dex 10^{-9} to 10^{-8}M	Sensitive	Resistant	Resistant
Dex 10^{-6} to 10^{-5}M	Sensitive	Sensitive	Resistant

*Summary of results published in reference 8.

This interpretation is, however, based on the comparison of two different cell lines, a lymphoma and a thymoma. While both cell lines, originated in Balb/c mice, are thymus derived and express the Thy 1 surface antigen, they have been isolated independently: the S49 tumor appeared in a lymph node after injection of mineral oil, and the W7 tumor in the thymus after x-irradiation. To support that model, hemizygous derivatives (r^+/r^-) of the W7 line, similar to the S49 line, had to be isolated. The selection for such putative W7 (r^+/r^-) hemizygotes was achieved on the basis of the observation that the S49 line is partially resistant to dexamethasone concentrations in the range of 10^{-9} to 10^{-8} M (see Table I). If this partial resistance is due to the lower receptor content of the S49 line, then selection for resistance to such low dexamethasone concentrations should yield the desired W7 (r^+/r^-) hemizygotes. Indeed, if a population of 8.8×10^7 W7 cells is exposed to 5×10^{-9} M dexamethasone, variants resistant to this low concentration of the steroid appear at a frequency of 3.3×10^{-7} [8]. The hemizygous (r^+/r^-) nature of these partially resistant W7 clones was confirmed by showing that, like the S49 line, they contain approximately 15,000 dexamethasone binding sites per cell, and they give rise to fully resistant variants at high frequencies of the order of 10^{-6} to 10^{-5} [8].

TYPES OF DEXAMETHASONE–RESISTANT VARIANTS

Since, as just described, variants of W7 can be obtained that are resistant only to low concentrations of dexamethasone (10^{-9} to 10^{-8}M), one should make the distinction between such partially resistant variants and variants selected for full resistance to high concentrations of dexamethasone (10^{-6} to 10^{-5}M). All types of variants either expected or isolated from the S49 and W7 lines are listed in Table II.

All dexamethasone-resistant variants derived from the S49 line, by us and by others [7, 13–17], result from receptor defects. Approximately 80–90% of the S49 variants are "receptorless' (r^-) in that they display essentially no dexamethasone-binding activity. One cannot, at this point, determine whether these variants lack the glucocorticoid receptor altogether or whether they contain a receptor with a greatly reduced affinity for the hormone. The availability of an anti-glucocorticoid receptor serum might allow, in the future, the detection in some of these "receptorless" variants of an altered receptor cross-reacting immunologically with a normal receptor.

Besides "receptorless" variants, the S49 line yields approximately 10–20% of the resistant clones that still contain receptor, although usually a reduced amount compared to the parental line [17]. Moreover, the receptors of these variants manifest various defects. Most of these altered receptors are "nuclear transfer defective" (nt^-) [7, 15, 17] in that a smaller percentage of these receptors undergo nuclear translocation than in the case of the parental line, although a few S49 variants have been found in which the extent of nuclear transfer is increased (nt^i) [14, 16]. Both nt^- and nt^i types of receptors manifest an altered affinity for DNA [14], as well as sedimentation properties and a behavior on gel permeation columns [16] different from those of normal receptor. Moreover, the receptor-steroid complexes of all the nt^- variants tested in our laboratory showed altered sensitivity of nuclear binding to ionic strength [17].

Resistant variants having normal receptor and resulting from a defect in another step of the killing mechanism have been named "deathless" (d^-) [7], but have, in fact, not been demonstrated. Two resistant S49 clones originally classified as d^- by Sibley and Tomkins [7] turned out, upon close examination by these investigators, to have detectable receptor defects. More recently, two other resistant S49 clones were found to have normal receptor [16], but the biochemical criteria used may have failed to reveal a subtle receptor alteration. To obtain further evidence for the d^- nature of variants, a genetic test of positive complementation should be used, showing that fusions between a d^- variant and receptor variants yield glucocorticoid-sensitive hybrids. Complementation analysis was not performed in the case of the two putative d^- variants having apparently normal receptor, because fusions between two S49 lines are extremely rare.

Approximately 100 variants resistant to high dexamethasone concentration were isolated from W7 (r^+/r^-) hemizygous lines described above and, as expected, gave rise to the same types of variants as the S49 line and in the same proportions: 84% were of the receptorless (r^-) phenotype, and all receptor-containing variants were of the nt^- type [17]. No nt^i or d^- variant was observed.

The fact that all resistant variants derived from the S49 line and from the W7 (r^+/r^-) line result from receptor defects is reasonable because of the presence of a single functional allele of the r gene in these lines. The homozygous W7 (r^+/r^+) line should, obviously, be used to search for variants of the d^- type: the presence of two copies of the r^+ allele would lower the frequency of receptor variants 10^6-fold, to a value on the order of 10^{-12}, and increase the probability of obtaining variants in another locus involved in the cytolytic response. However, when a total of 127 variants resistant to high concentrations of dexamethasone were derived from the W7 (r^+/r^+) line after mutagenesis by N-methyl-N′-nitro-N′-nitrosoguanidine, ethyl methanesulfonate, or ultraviolet light, all turned out to result from receptor defects as well [11]. The absence of deathless variants derived from the W7 (r^+/r^+) line is puzzling, and several possible explanations for that observation will be considered in the discussion.

As far as the variants selected from the W7 (r^+/r^+) line at low dexamethasone concentration are concerned, we found, besides the hemizygotes W7 (r^+/r^-) described above, a few variants that appear to have an altered receptor with a reduced affinity for glucorticoids (r^a). Such variants are not expected at dexamethasone concentrations high enough to compensate for that reduced affinity. One could also have expected, at low steroid concentrations, to select for variants with a reduced permeability to dexamethasone, if a protein is involved in the transport of the steroid. Although it has generally been assumed that glucocorticoids and other steroids enter target cells by simple diffusion by virtue of their lipophilic nature, this may not be the case for all cell types. The available evidence for

TABLE II. Types of Dexamethasone-Resistant Variants

Selective dex concentration	Type	Name	Dex affinity	Nuclear transfer	Salt extraction from normal nucleus	Salt elution from DNA cellulose	Observed	References
High[a]	Receptor negative	r^-	Not detectable				S49, W7	[7, 10, 16]
High[a]	Altered receptor	nt^-	Normal[b]	Reduced	Altered	Altered	S49, W7	[7, 10, 13–16]
High[a]	Altered receptor	nt^i	Normal[b]	Increased	Altered	Altered	S49	[13, 15]
High[a]	?	d^-	Normal[b]	Normal[d]	Normal[d]	Normal[d]	(S49) ?	[7, 15]
Low[c]	Altered receptor	r^a	Reduced	Normal[d]	Normal[d]	Normal[d]	W7	e
Low[c]	Reduced amount of normal receptor	r^+/r^-	Normal[b]	Normal[d]	Normal[d]	Normal[d]	W7	[8]
Low[c]	Dex permeability		Normal[b]	Normal[d]	Normal[d]	Normal[d]	no	

[a] 10^{-6} to 10^{-5}M.
[b] $K_d = 1.3 + 0.03 \times 10^{-8}$M [12].
[c] 10^{-9} to 10^{-8}M [12].
[d] Indistinguishable from the behavior of the parental receptor.
[e] Bourgeois and Newby, unpublished result.

active steroid transport systems has recently been reviewed [18]. The isolation of variants resistant to low dexamethasone concentrations but containing a normal amount of receptor having normal affinity for the steroid could provide valuable genetic evidence for the existence of a transport mechanism. However, no such variant was observed.

STUDIES WITH CELL HYBRIDS

The analysis of cell hybrids can give further insight into the nature of the receptor defects leading to resistance. In particular, our results have been interpreted in terms of a single genetic locus, r, coding for the glucocorticoid receptor or a receptor subunit, which could be present as a normal active allele r^+, as an inactive allele r^-, or in an altered allelic state such as r^{nt}. Two alternative models should, however, be considered. One is the existence of two distinct loci coding for non-identical subunits of the receptor, which could each be inactivated or altered independently. Another possibility is that the receptor could be inactivated by defects in a locus coding for a receptor "activation" protein such as a receptor-specific kinase [19]. Both these models involve at least two different genes that would be necessary for receptor activity and, therefore, predict that hybrids between different receptor-defective variants should acquire glucocorticoid sensitivity by positive complementation. Another possibility that can be examined in cell hybrids is that the receptor is an oligomer made up of several identical subunits encoded by the r gene. In that case, the altered receptor product of some r^{nt} or r^- alleles could, in a hybrid, associate with normal subunits to yield an inactive receptor oligomer. This could be detected by the fact that a hybrid between a sensitive cell (r^+) and a resistant variant (r^{nt} or r^-) would be resistant because of negative complementation.

An extensive genetic analysis of the S49 glucocorticoid-resistant variants has been difficult, because S49 lines fuse very poorly among themselves [17], although some S49 × S49 hybrids have been described [16]. Such analysis became possible by using the W7 line and its variants, because W7 lines fuse readily with S49 lines at frequencies of 10^{-4} to 10^{-5}, and W7 × W7 hybrids can be obtained as well, although at somewhat lower frequencies on the order of 10^{-6} [20–22]. Thanks to the presence of either bromodeoxyuridine or thioguanine resistance markers in these lines, hybrids can conveniently be selected in hypoxanthine-aminopterine-thymidine medium [23].

A variety of dexamethasone-resistant r^- and nt^- lines were fused among themselves and with the parental sensitive line [20]. Neither positive nor negative complementation was observed: the dexamethasone-sensitive phenotype was always dominant over resistance. These results do not rule out the existence of the different genes necessary for active receptor or an oligomeric structure of the receptor, but they do not support such models. These studies have revealed two rather unexpected characteristics of these hybrids: First is the fact that the amount of receptor produced in a hybrid clone can vary largely and is not always the sum of the receptor content of the parental lines. The second observation is that such hybrids give rise to dexamethasone-resistant clones at frequencies orders of magnitude higher than expected on the basis of the ploidy of the r^+ gene.

The characteristics of some hybrids between W7 lines that are either homozygous (r^+/r^+ or r^-/r^-) or hemizygous (r^+/r^-) at the receptor locus are shown in Table III. These particular hybrids contain one, two, three, or four copies of the r^+ allele; their glucocorticoid receptor is normal and present in an amount that reflects the r^+ gene dosage effect. The frequencies of resistant variants derived from these hybrids are also listed in Table III. In the case of the hybrid containing a singly copy of the r^+ allele, a frequency of approxi-

TABLE III. Characteristics of W7 Parental and Hybrid Clones

Receptor alleles	Number of r^+ alleles per cell	Receptor sites per cell ± SE[a]	Frequency of Dex^r[b]
r^+/r^+	2	30,000 ± 3,000	$< 1.2 \times 10^{-10}$
r^+/r^-	1	15,000 ± 1,500	9.6×10^{-6}
r^-/r^-	0	< 100	1
$r^+/r^+ \times r^+/r^+$	4	57,500 ± 3,900	$< 2.5 \times 10^{-8}$
$r^+/r^+ \times r^+/r^-$	3	42,800 ± 1,800	$< 7.1 \times 10^{-9}$
$r^+/r^- \times r^+/r^-$ or $r^+/r^+ \times r^-/r^-$	2	32,500 ± 1,500	7.6×10^{-6}
$r^+/r^- \times r^-/r^-$	1	12,700 ± 1,500	1.6×10^{-3}

[a]Mean value ± SE calculated from a series of independent determinations published elsewhere [21].
[b]Data published elsewhere [22].

mately 10^{-3} was observed, three orders of magnitude higher than in the case of the diploid cell, r^+/r^-, containing a single r^+ allele. Similarly, the frequency obtained in the case of the hybrid containing two r^+ alleles is 7.6×10^{-6}, orders of magnitude higher than the value of $< 1.2 \times 10^{-10}$ observed for r^+/r^+ diploid cells. These very high frequencies were shown to result from segregation of the r^+ allele(s) [20] accompanied by the loss of chromosomes [22]. This behavior of hybrids most likely is responsible for the observation mentioned earlier that the frequency of dexamethasone-resistant variants of S49 was the same in diploid or pseudo-tetraploid S49 cells [5–7]. Because of segregation-like events, the frequency of variations to dexamethasone resistance behaves as if it were independent of ploidy when diploid cells containing a single r^+ allele are compared to tetraploid cells containing two r^+ alleles.

The hybrids listed in Table III have also been used to show a tight correlation between glucocorticoid receptor content and cytolytic response [21, 22]. The hybrid containing a single dose of receptor (12,700 ± 1,500 receptor sites per cell) is the least sensitive to the killing effect of dexamethasone, and sensitivity increases with the number of receptors per cell. Such a correlation between number of cellular glucocorticoid receptors and clinical response has been sought in the case of human leukemias and lymphomas, in the hope of using receptor measurements to predict the outcome of glucocorticoid therapy. In the case of human leukemias, recent studies find little correlation between receptor levels and clinical response or glucocorticoid sensitivity of cells in vitro [24, 25]. The W7 model system, which consists of a homogeneous population of cloned and exponentially growing cells, is likely to be more favorable than clinical samples of lymphocytes to demonstrate such a correlation. However, the lymphocytolytic response in mice, a glucocorticoid-sensitive species, may not be directly comparable to the inhibitory effects of glucocorticoids in humans, and recent evidence has been obtained that glucocorticoids can affect T-cell proliferation indirectly by inhibition of T-cell growth factor production [26]. Whatever the effects responsible for the therapeutic benefits of glucocorticoids in humans, these could be mediated by glucocorticoid receptors, and the tight correlation between receptors and response observed in this simple model system is encouraging.

DISCUSSION

The analysis of glucocorticoid-induced lymphocytolysis in murine lymphoid cell lines points to some potential problems to be aware of when using a somatic cell genetic approach to hormone action. In particular, one cannot take for granted that somatic cell lines established in culture are functionally diploid at every locus. Partial hemizygosity can be expected and has been observed [27] in some aneuploid cell lines, which do not contain the normal diploid number of chromosomes and show, upon karyotyping, many chromosomal rearrangements and translocations. Our data indicate, however, that functional hemizygosity can also occur in a pseudo-diploid line, like S49, which appears to have a normal and stable diploid complement of 40 chromosomes. No chromosome banding studies have been done in this cell line to examine possible chromosome rearrangements.

It is probable that, in the case of the S49 line, the inactivation of one of the two copies of the r^+ gene is the result of a mutation rather than of a chromosome rearrangement. Whatever the mechanism of r^+ inactivation, the S49 hemizygote must have been selected because of its resistance to low concentrations of glucocorticoids. This selection could have occurred in the animal, or in tissue culture, in the course of numerous transfers of the S49 in media with serum containing low levels of glucocorticoids. In general, the possibility of unwanted selection of variants with an altered sensitivity to one of the hormones present in the serum of tissue culture media should be kept in mind. In addition, selection for variants independent of a hormone for growth could occur upon transfers in medium with a serum deficient in that hormone.

The segregation-like events that seem to occur in cell hybrids introduce another complication in the genetic analysis of variants. These events, by increasing the apparent frequencies of variants arising from hybrids, may obscure the results of complementation or dominance tests.

These studies have yielded a collection of receptor variants that will, no doubt, be useful for biochemical studies of the mode of action of these receptors: in fact, this is the only steroid-mediated system in which such variants are available. These have recently been employed by Johnson and Baxter [28], who were able to show in the S49 line a glucocorticoid-induced alteration in chromatin structure as detected by a change in the number of initiation sites for Escherichia coli RNA polymerase. This glucocorticoid effect on chromatin was not observed in two dexamethasone-resistant variants of S49, an r^- and an nt^i.

The absence of deathless variants, blocked at a step beyond the receptor, deserves some comments. In the case of S49 or W7 (r^+/r^-) cells, it is understandable that the isolation of such variants would be extremely unlikely, because the hemizygosity of the r gene strongly favors the isolation of resistant variants resulting from defects at that locus. In the case of the W7 (r^+/r^+) line, however, the presence of two r^+ alleles, which should be a necessary condition to obtain deathless variants, was not sufficient to observe a single d^- among 127 variants examined [11]. At least four explanations of this result should be considered. It could simply mean that deathless variants are > 100 times less frequent than receptor variants and that a larger collection of variants should be analyzed. A second possibility is that the other functions involved in the lytic response are vital to the cell, and that defects in these functions are, therefore, lethal. It is also conceivable that the interaction of the receptor with the nuclear acceptor triggers two (or more) independent pathways, both leading to cell death. In that case, at least two different functions would have to be blocked simultaneously, by mutations -- a very low probability event. Finally,

one should keep in mind the possibility that the receptor itself directly induces the lytic process; in that case, no other function would be involved and no new type of variant can be expected. Obviously, more work is needed to explore these alternatives.

The availability of deathless variants would be essential to analyze this system further, because they could provide a handle on the biochemical analysis of the lytic mechanism. One limitation of the lymphocytolytic system is the fact that the mechanism responsible for cell death is entirely obscure. No new mRNA or protein is known to be induced by glucocorticoids in these cells. A number of studies, recently reviewed by Munck and Leung [29], showing that many effects of glucocorticoids on sensitive lymphoid cells can be blocked by inhibitors such as actinomycin D and cycloheximide, suggest that RNA and protein synthesis may be required for the cytolytic response. Recently, these inhibitors have indeed been shown virtually to abolish the glucocorticoid-mediated lysis of rat thymic lymphocytes, as measured by ^{51}Cr release [30]. However, the identification of an mRNA or a protein induced by the steroid remains to be done, and this would be an important advance, because it would yield a biochemical assay for the response and could allow cloning of the glucocorticoid-induced gene (s). An ideal system to study hormone action in general, and the mode of action of steroid receptors in particular, would be one in which a known mRNA is induced in large amounts, coding for a protein that allows an easy selection for unresponsive variants. Unfortunately, no such system is available at this time.

This model system has provided the first clear evidence for a tight correlation between the glucocorticoid receptor content of cells and the response to the steroid. In spite of the fact that the mechanism of the lymphocytolytic response is unknown, these results indicate that these receptors are not present in excess in lymphoid cells and suggest that other responses, which may contribute to the immunosuppressive or antileukemic effects of glucocorticoids, may be limited by these receptors as well.

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