Title
Frequency and mechanism of resistance in variants of steroid sensitive cultured mouse lymphoma cells

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Publication Date
1974

Peer reviewed|Thesis/dissertation

FREQUENCY AND MECHANISM OF RESISTANCE IN VARIANTS OF STEROID SENSITIVE CULTURED MOUSE LYMPHOMA CELLS

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

in the

GRADUATE DIVISION

(San Francisco)

of the

UNIVERSITY OF CALIFORNIA

[redacted]

Date Librarian

Degree Conferred: 01 JAN 1974

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January 1974

ABSTRACT

Frequency and Mechanism of Steroid Resistant Variants in a Glucocorticoid Sensitive Population of Cultured Mouse Lymphoma Cells

Cells of the cultured mouse lymphoma line, S49, are killed by physiological concentrations of adrenal corticosteroids. In soft agar these cells clone with an efficiency of 0.5 to 1.0 in the absence of steroid, but at 2×10^{-5} in the presence of 5×10^{-7} M Dexamethasone (dex), a synthetic adrenal steroid. Those colonies which grow in the presence of the hormone are composed of steroid-resistant cells. Luria-Delbrück Fluctuation Analysis showed that the development of resistant variants is a random event, independent of the selective conditions. Using the Lea and Coulson Maximal Likelihood Method, the rate of appearance of resistant variants/cell/generation was calculated to be 3.5×10^{-6}.

Treatment of a sensitive clone with the mutagens nitrosoquanidine (2×10^{-5} M) and 9-aminoacridine (2×10^{-5} M) raised the frequency of resistant cells 50- to 100-fold and 20-fold respectively. These results are consistent with, but do not establish that the transition from steroid sensitivity to resistance is a mutation. What ever their origin, the resistant clones are rare, stable, heritable variants which do not appear to differ from the sensitive cells in any trait except steroid-

sensitivity. The first step in the series of events leading to cell death appears to be the association of the steroid with a single class of high-affinity cytoplasmic glucocorticoid receptor molecules; this is followed by localization of the steroid-receptor complex in the nucleus and presumably in macromolecular synthesis resulting in the induction of a "suicide protein" which kills the cell. Forty-two steroid-resistant clones were isolated from the sensitive population. When measured in whole cells, thirty-three of these lacked steroid-binding activity, eight had activity in the range of the sensitive control and one displayed intermediate activity. Cell-free studies of the binding of dex to the specific receptors using a particle-free supernatant confirmed that the first class lacked receptor activity; these cells were designated, steroid resistant, "receptorless", $S^R(r-)$. Extracts of those cells which exhibited normal steroid binding also displayed normal receptor activity and were designated steroid-resistant, receptor-containing, $S^R(r+)$. These receptor-containing resistant clones were further tested for their ability to localize the receptor-steroid complex in the nucleus. Three of the eight clones tested showed impaired localization of steroid in the nucleus; these were designated nuclear transfer minus, $S^R(r+nt-)$. The remaining clones which bound the steroid in the cytoplasm and translocated it to the nucleus, but were nonetheless resistant to steroid, were designated "deathless", $S^R(r+nt+d-)$. Studies in which the steroid-resistant clones were isolated from steroid-sensitive clones, both pseudo-diploid, like the wild population and a pseudo-tetraploid gave

very similar results. Cell-free studies of the binding of steroid to extracts of nuclear-transfer-minus clones demonstrated that the receptor activity was indistinguishable from normal controls. In contrast, extracts of "deathless" clones exhibited a somewhat higher affinity for steroid and greater concentration of receptor molecules per cell than the control clones. Detailed biochemical analysis of the mechanism of steroid resistance in these clones will allow us to determine the pathway of hormone response in these cells. Since the mechanism of the steroid-response appears to be very similar in all tissues surveyed, the results would be expected to be relevant to these systems.

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ACKNOWLEDGEMENTS

I would like to thank John Baxter, Robert Ivarie, Toni King, Barbara Levinson, Guy Rousseau and Kieth Yamamoto, each of whom made important contributions to this work.

To Philip Ballard and Ulrich Gehring go special thanks for their interest in this work as it progressed, for thoughtful discussion of the results and for careful reading of the manuscript, all of which improved greatly the final product.

I would like to thank my thesis committee, Brian McCarthy and David Martin for taking the time to read and discuss the manuscript.

To my advisor, Gordon Tomkins, I owe a large debt for his continuing time, support and stimulation throughout the course of my graduate career.

Lastly, I would like to thank Tom Sibley, Philip Ballard and Roberta Ballard, without whom none of this work would ever have been finished.

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CHAPTER ONE

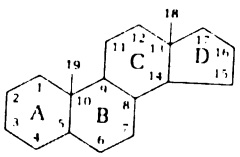
INTRODUCTION

Steroid hormones constitute a well-defined group of vertebrate hormones whose basic structures are derivatives of the tetracyclic carbon skeleton in Fig. 1. On the basis of both chemical substitution at various positions on the basic ring structure and physiological effects they can be subdivided in mammals into estrogens, androgens, progestins, glucocorticoids, mineralocorticoids and vitamin D. Early work described the complex physiological effects of these steroids on whole animals, but the diversity of the responses and the interaction of the steroids with other hormones made investigation of their molecular mechanism extremely difficult (for reviews of the chemistry and physiological effects of the various groups of steroids see Pincus, et al., 1948-1964).

The resolution of these experimental difficulties began with the observation by Jensen and Jacobsen (1960, 1962) that radioactive estradiol is selectively concentrated in immature rat uterus, one of the organs which exhibits a vigorous physiological response to this hormone. Subsequent work by Jensen's group and others established that the uterus and other estrogen target organs like vagina and the anterior pituitary demonstrated a striking affinity for estradiol either when it was administered to an intact animal (Jensen and Jacobsen, 1962; Glascock and Hoekstra, 1959; Stone et al., 1963) or to isolated target organs or tissues (Stone and Bagget, 1965; Jensen et al., 1966; Terenius, 1966).

Figure 1.

The carbon skeleton common to all steroids.


18
19
A
B
C
D

Work on other steroids and their target tissues established similar localization of hormone in these systems: aldosterone and kidney or toad bladder, (Fanestil and Edelman, 1966; Sharp and Alberti, 1970); progesterone and chick oviduct (O'Malley et al., 1970; Sherman et al., 1970); dihydrotestosterone and ventral prostate (Anderson and Liao, 1968; Bruchovsky and Wilson, 1968); cortisol and liver (Beato et al., 1970); cortisol and thymus (Munck and Brinck-Johnsen, 1968); vitamin D and intestine (Lawson et al., 1968).

By the end of 1970, it had become evident that despite the marked differences in physiologic response to the various steroids, the earliest events in steroid hormone-target tissue interaction were remarkably similar in all of these systems (for an extensive discussion see Raspé, 1970). First, the particle-free supernatant fraction of target tissues was shown to contain specific high-affinity steroid-receptor molecules. These receptors were identified with remarkable ease in cytoplasmic extracts from uterus (Toft and Gorski, 1966; Jensen et al., 1968), chick oviduct (O'Malley et al., 1969; Sherman et al., 1970), ventral prostate (Fang et al., 1969; Baulieu et al., 1970), kidney (Herman et al., 1968), liver (Beato et al., 1970), thymus (Munck and Wira, 1970) and intestine (Tsai and Norman, 1973; Brumbaugh and Haussler, 1973). In fact, a systematic study of rat tissues showed that specific glucocorticoid receptors could be identified in all tissues in which a physiologic response had been previously demonstrated (Ballard et al., in press).

That the next step in the action of the hormone was nuclear localization was established using two approaches, autoradiography and

and nuclear isolation. In either case, when animals or tissues were treated with radioactive hormone at temperatures between 20 and 37°, the hormone localized over the nuclei in the intact cells (Stumpf and Roth, 1966; Edelman et al., 1963), or in isolated nuclei when cells were fractionated in uterus (Jensen et al., 1968; King et al., 1965; Maurer and Chalkley, 1967), ventral prostate (Liao et al., 1970; Baulieu et al., 1970; Tveter, et al., 1970), chick oviduct (O'Malley et al., 1970), kidney (Swanek et al., 1970), toad bladder (Ausiello and Sharp, 1968), liver (Beato et al., 1970), thymus (Munck et al., 1972), and intestine (Tsai and Norman, 1973). In all cases where it was attempted, the hormone could be extracted from the isolated nuclei still complexed with a protein using high ionic strength (usually 0.3 M salt).

This two step mechanism suggesting that nuclei are the ultimate target of the hormone, raised speculation that the steroids were eliciting their responses by affecting transcription and/or translation in the target cell. That new macromolecular synthesis is required for the response of a tissue to its hormone was largely inferred from the fact that specific inhibitors of RNA and protein synthesis (usually actinomycin D and cycloheximide or puromycin) did not inhibit hormone binding to cytoplasmic receptors or nuclear localization but still abolished the hormone response in uterus (Jensen, 1965), toad bladder (Edelman et al., 1963), thymus (Munck, 1971), chick oviduct (O'Malley et al., 1969), and liver (Holt and Oliver, 1969). The new RNA or protein molecules were not isolated or identified in any of these systems.

One of the drawbacks of working with hormone-treated animals or

tissues is that the interaction between different organ systems, effects of other hormones and the diversity of cell types in tissues often seriously complicate the identification and analysis of the primary hormone response (for an example of one such problem, see Williams and Gorski, 1971). Simpler systems consisting of a single responsive cell type circumvent some of these difficulties. Several such systems have been established in culture, including glucocorticoid responsive mouse fibroblasts (Pratt and Aronow, 1966), lymphoid cells (Horibata and Harris, 1970) and several hepatoma lines (Thompson et al., 1966; Pitat et al., 1964). The relative simplicity of these systems made possible detailed studies of the very early and very late events in the hormone response. As in the tissues earlier investigated, the basic two-step mechanism of early hormone action, cytoplasmic binding followed by nuclear localization, was described in these cell lines (Pratt and Ishii, 1972; Baxter and Tomkins, 1970,1971; Baxter et al., 1971). In the hepatoma cells the induction of liver-specific enzymes by both natural and synthetic glucocorticoids has been studied in great detail. Combining a great many results, including those obtained with inhibitors of macromolecular synthesis, a model of the late "post nuclear" events in the induction of one of these enzymes by glucocorticoids was formulated (Tomkins et al., 1969). Because of the simplicity of the system and the ease with which hormones, inhibitors and radioactive substrates can be added or removed, testing of this model has been a particularly fruitful and controversial area (Lee et al., 1970; Reel and Kenney, 1968; Reel et al., 1970; Tomkins et al, 1969).

By 1970, the broad outlines of the mechanism of steroid hormone action had been identified in a wide variety of systems, including whole animals, isolated organs, tissues and cultured cells. Since each system had its particular strengths and weaknesses, the information was uneven. Nevertheless, a working hypothesis incorporating most of the results could be formulated as in Figure 2 (from Baxter and Forsham, 1972). While this diagram depicts a glucocorticoid response, a similar outline would describe the action of other steroids, by simply changing the physiologic response. The generality of this outline suggested specific questions which were explored from several different approaches in the late 1960's and early 1970's:

1. What is the nature of the interactions between the steroid and its cytoplasmic receptor and what are the properties of the complex?

2. What reactions (if any) are required for the receptor-steroid complex to localize in the nucleus?

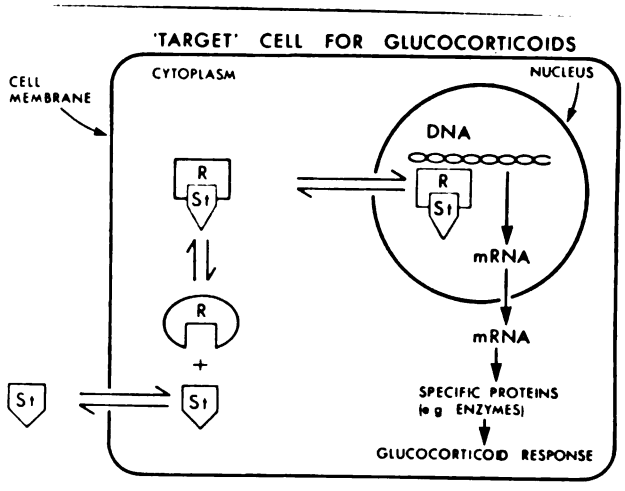
3. Where does the receptor-steroid complex bind in the nucleus? Are there specific sites for the binding reaction?

4. What new molecules are specifically made as the result of the receptor-steroid-nuclear interaction? What is their effect on the target cell?

The first question was pursued by several means. In the estrogen and progesterone systems the physical properties of the receptor-steroid complex were extensively examined. In particular its sedimentation velocity on sucrose gradients under various ionic conditions, its behavior when isolated from the cytoplasmic and nuclear fractions

Figure 2.

A diagrammatic representation of the early events in glucocorticoid hormone action (from Baxter and Forsham, 1972).


'TARGET' CELL FOR GLUCOCORTICOIDS
CELL MEMBRANE
CYTOPLASM
NUCLEUS
DNA
R
St
mRNA
mRNA
SPECIFIC PROTEINS (e g ENZYMES)
GLUCOCORTICOID RESPONSE

and its conversion from one form to another were determined. These have been thoroughly discussed in the Schering Symposium (Brecher et al., 1970; King et al., 1970; Alberga et al., 1970; Puca et al, 1970; Erdos et al., 1970; Jungblut et al., 1970; O'Malley et al., 1970) and in other publications (Yamamoto and Alberts, 1972). The groups agree that the receptor steroid-complex from the cytoplasmic fraction has a sedimentation velocity of about 4s in sucrose gradients with inoic strengths greater than 0.15 M, and about 8-9s in gradients of lower ionic strength (Toft and Gorski, 1966), presumably as a result of aggregation. The receptor-steroid complex isolated from nuclei has a sedimentation velocity of about 5s (Jensen et al., 1968). There is circumstantial evidence that the 5s form is the 4s molecule which has either acquired additional mass or changed shape (Yamamoto and Alberts, 1972), suggesting that the hormone is transported from the cytoplasm to the nucleus bound to the same molecule. Similar observations on sedimentation behavior in sucrose gradients have been made using the steroid receptors from rat kidney (Edelman, 1970), ventral prostate (Liao et al., 1970), chick oviduct (O'Malley et al., 1970), liver (Beato et al., 1970).

The structure-activity relationships of various steroids with regard to binding to cytoplasmic receptors and to ability to elicit a physiologic response yielded detailed information on this aspect of receptor function in several systems (Samuels and Tomkins, 1970; Rosseau et al., 1972; Roseneau et al., 1972; Edelman, 1970). Using the hepatoma tissue culture system, and a series of physiologically

active and inactive glucocorticoids, a model of the binding protein as an allosteric molecule whose configuration and subsequent physiologic effectiveness depends on the steroid present was developed (Samuels and Tomkins, 1970; Rousseau et al., 1972). Further insights into receptor function have been sought in attempts to purify the receptors by classical methods (Jensen et al., 1971; Hackney and Pratt, 1971; Mainwaring and Irving, 1970; Tu and Moudrianakis, 1973) or more recently by affinity chromatography of cell extracts by steroid-conjugated matrices (Sica et al., 1973). The extremely low concentration of receptors and their apparent tendency to aggregate have made this a difficult task.

The second question raised by earlier observations involved "activation" of the receptor-steroid complex; this was postulated to be a specific change, or set of changes, in the complex prerequisite for transfer to the nucleus or to binding to nuclear sites. In most systems the transfer reaction is temperature sensitive, occurring readily at 20°, but extremely slowly (on the order of more than 20 hours) at 0° (Munck and Wira, 1970; Mosher et al., 1971; Baxter et al., 1972; Jensen et al., 1968; Edelman, 1970). It was, however, reported to proceed readily at 0° in the progesterone system (O'Malley et al., 1970). In all of these systems specific nuclear binding requires that the hormone be first bound to the cytoplasmic receptor; free steroid does not specifically bind to nuclear sites. Since, in the several systems, it is the 5s form which is extracted from the nucleus, and the 4s form which is found in cytoplasmic extracts, it

is conceivable that the transition from 4s to 5s might correspond to "activation". Unfortunately, there is no information on this point. Furthermore, the correlation of these sedimentation velocity changes with the process called "activation" in the glucocorticoid responsive HTC cells (Higgins et al., 1973) has not been made. It does appear, at least, that binding of the hormone to the cytoplasmic form of the receptor is prerequisite to nuclear localization and that some alteration in or additions to the receptor-steroid complex must be made before it migrates to the nucleus.

Considerable information has accumulated which pertains to the fourth question, as well. The earliest observations on overall physiological effects of the various steroids were the foundations for this work. As noted previously, new macromolecular synthesis is required for the action of the hormone, in all cases studied. These sorts of experiments were continued and extended to include some very sophisticated uses of inhibitors of RNA and protein synthesis (Katzenellenbogen and Gorski, 1972; Tomkins et al., 1969; Scott et al., 1972; Reel et al., 1970; Lee et al., 1970; Munck et al., 1972; Makman et al., 1971). The steroids have been shown to cause changes in patterns and amounts of RNA (Garren et al., 1964; Gorski et al., 1965; Korner, 1965; Williams-Ashman, 1965; Kenney et al., 1965) which are reflected in new and/or increased protein synthesis (Scott et al., 1972; O'Malley and McGuire, 1968; O'Malley and McGuire, 1969; Schutz et al., 1973; Rhoads et al., 1973; Chan et al., 1973).

Deciphering which of the observed changes are primary hormone effects, and which are of secondary consequence is extremely difficult in most systems. Only in the hepatoma system is the induced change well-defined and free from the complication of the large overall increases in RNA and protein synthesis seen in growing and developing systems like uterus. Even in the case of the hepatoma cells, the induced protein represents only 0.1% of the cell total, so far frustrating efforts to isolate its messenger (Scott et al., 1972). In one system, the estrogen-induced differentiation of chick oviduct, the response to the hormone is the production of egg proteins, one of which is ovalbumin. Here, the induced protein constitutes more than 60% of the total synthesis; its messenger and those for avidin, and other egg proteins have been isolated, purified and translated in a heterologous in vitro protein synthesizing system (Palacios et al., 1973; Palmiter, 1973; Palmiter and Smith, 1973). In this one case the hormone is clearly increasing the number of messenger RNA molecules available for translation. The immunologic methods outlined by these workers may allow the isolation of specifically induced messengers from other hormone-responsive systems in which the new proteins comprise very small fractions of the total. Further analysis of the oviduct system should yield additional information on the mechanism of this increase in messenger RNA and allow a rigorous test of the model proposed earlier (Tomkins et al., 1969).

Although there are many gaps in the available information, there is general agreement on the outlines of the answers to the first, second

and fourth questions. Furthermore, available biochemical techniques seem adequate to the task.

There remains one large question to be answered: what is going on in the nucleus? While the answers to the other question are generally rather uniform from one system to another, in this case the disparity from system to system, even laboratory to laboratory is great. Based on analogy to prokaryotic models, it is attractive to suppose that the receptor-steroid complex, after cytoplasmic "activation" binds specifically to sites on the DNA. Since, in mammalian cells, the DNA is extensively complexed with both histone and acid proteins, it is quite reasonable that these might also contribute some specific components to the interaction. Experiments designed to approach this question have made three rather different sorts of observations. O'Malley's group, investigating the induction of avidin by progesterone in chick oviduct and estrogen effects on uterus, find that the receptor-steroid complex binds to DNA with little or no specificity, but that a particular acidic protein fraction from oviduct chromatin (and not chromatin from other tissues) confers specificity to the interaction (Spelsberg et al., 1972; Spelsberg et al., 1971; O'Malley et al., 1972, Steggles et al., 1971) i.e., the receptor-steroid complex binds "specifically" to chromatin from oviduct, but not to chromatin from other sources. Other workers using the estrogen-uterus system, found that the binding of receptor-steroid complex to nuclei is an extremely high capacity-low affinity interaction (Williams and Gorski, 1972), quite the reverse of the O'Malley

group. Yamamoto and Alberts (1972) measured the binding of receptor-steroid complex to isolated DNA and also concluded that the interaction is one of high capacity and low affinity. Since the response curves of uterus to increasing doses of estrogen demonstrate that the physiologically important reactions occur at very low hormone concentrations, these observations present a clear paradox. These authors suggest that a small subset of the observed nuclear or DNA binding may represent the physiologically relevant sites (Yamamoto, 1973; Williams and Gorski, 1972). How these sites can be identified among the apparently huge excess of "nonspecific" sites is an unsolved problem.

Still a third group investigated the binding of receptor-steroid complex to isolated nuclei and DNA in the glucocorticoid-responsive HTC cells (Baxter et al., 1972; Higgins et al., 1973; Rousseau et al., in press). They found that the binding reaction in both cases is a high affinity-low capacity interaction. In addition, in their system, binding of the complex to DNA exceeded binding to chromatin, the reverse of the finding in chick oviduct (Spelsberg et al., 1971). Moreover, these investigators reported that in these cells, the binding of receptor-steroid complex to nuclei in whole cells did not compete with further binding of receptor-steroid complex to the same nuclei <u>in vitro</u> (Higgins et al., in press).

Reconciling all of these observations is obviously a difficult task and one made doubly difficult by the uncertainties surrounding the structure of chromatin in eukaryotic cells (for an extensive review

see Huberman, 1973). The structural integrity of isolated chromatin and the chromatin in isolated nuclei has been questioned, since the ionic conditions of isolation certainly differ from those *in vivo*. The ionic strength of the isolation buffer is clearly a critical factor in determining the accessibility of the DNA to added ligands and to the binding of the ligands, making interpretation of experiments of this sort extremely difficult and controversial (for examples see Clark and Felsenfield, 1971; Pederson, 1972; Mirsky, 1971).

In the light of these problems, it would appear that determination of the sites of nuclear binding by receptor-steroid complex by strictly biochemical methods may be a long term process. For this reason, we have begun to combine a more genetic approach with classical biochemical analysis. The goal of such a study was to isolate a series of cells whose response to steroid is abberrant, and by characterizing the various defects to determine the molecular events required for a normal hormone response. This approach is a familiar and powerful one in prokaryotic systems, but has only recently become feasible in eukaryotic cells other than yeast. Prior to the development of tissue culture, the genetics of mammalian systems was confined to whole animal systems, requiring in most cases that the experimenter characterize whatever anomalies had occurred naturally. In the case of hormone responses, viable disturbances are extremely rare, since unresponsiveness is usually lethal. An exception to this is testicular feminization, a condition in which genetic males are phenotypically female due to an inability to respond to androgens. It has been shown

in mice (and inferred to be the case in humans) that this defect is the result of a defective androgen receptor which renders target tissues unresponsive to the hormone (Gehring et al., 1971). In addition, several kinds of glucocorticoid-sensitive malignancies have been shown to become hormone-unresponsive, with a concommitant loss of hormone receptor activity (Lippman et al., 1973; Kirkpatrick et al., 1971). While these add important information, the fact that the populations tested are heterogeneous mixtures of cells, limits the scope of the conclusions.

Growing individual cells in culture allows easy manipulation of large homogeneous populations of cells, selection of rare abberrant cells, mutagenesis and isolation of the progeny of a single cell. As noted previously, several hormone-responsive cell lines had been fruitfully employed in the biochemical investigation of steroid action. Extension of this work employing genetic techniques added a second dimension to their usefulness. This work was begun using the glucocorticoid-responsive hepatomas (Thompson and Gelehrter, 1971; Weiss et al., 1972; Weiss and Chaplain, 1971) and lymphoid lines (Horibata and Harris, 1970; Harris, 1970; Gehring et al., 1972). Because of the relative difficulty of selecting aberrant cells in the hepatoma lines, we chose one of the glucocorticoid-responsive lymphoma lines, S49 (Horibata and Harris, 1970) for detailed analysis. In these cells the response to physiologic doses of glucocorticoids is cell death (Harris, 1970). Therefore, selection of cells with an impaired response to hormone simply required isolation of the survivors of

hormone treatment; biochemical analysis of these cells allowed identification of their specific defect in hormone responsiveness. Experiments with rat thymocytes implied that their death in response to glucocorticoids required both RNA and protein synthesis (Munck et al., 1972; Makman et al., 1971). This would suggest that the mechanism of steroid action in these cells may be similar to that in the previously described systems, making the S49 cells a good model system for studying hormone action.

Earlier work on this cell line had demonstrated that steroid-resistant lines could be obtained with remarkable ease by treating large populations of sensitive cells with gradually increasing concentrations of the synthetic glucocorticoid, dexamethasone (Baxter et al., 1971; Roseneau et al., 1972). In order to collect a series of steroid-resistant cells with different defects in their hormone response, selection has to be effected in a single step and allow the isolation of the progeny of a single cell. The first part of the work reported here describes a technique which fulfills these requirements. The cells isolated are shown to be stably resistant to steroid; the development of the resistance is demonstrated to be a rare, random event.

The detailed biochemical analysis of these isolated steroid-resistant cells comprises the second major part of the study. The information presented demonstrates that at least three distinct steroid-resistant phenotypes can be identified in these cells. Examination of the abberrant cells is an independent means of establishing which of the previously observed steroid-cell interactions are required for the

action of the hormone. This approach will, we hope, be of particular value in clarifying the events which must occur in the nucleus since it is here that the presently available information seems least satisfactory.

CHAPTER TWO

MATERIALS AND METHODS

Cells

The cell line, S49.1T.B4 was obtained from Ruth Epstein of the Salk Institute. It is a derivative of a steroid-sensitive lymphoma, S49, induced by mineral oil in a Balb/c mouse by Horibata and Harris (1970). It carries θ and TL antigens on its surface and is resistant to 10^{-4} M BUdR. These cells were first cloned in 1970 to produce S49.1T.B4.1A, abbreviated in the text as S49.1A. Individual sub-clones are identified by additional numbers, e.g., S49.1A.1, S49.1A.2, etc.; those which are steroid-resistant are further identified with the letter "R": S49.1A.1R, S49.1A.2R, etc.

Cells were counted either in a hemocytometer or with a Coulter Counter (Coulter Electronics, Hialeah, Fla.). Cell viability was determined by the trypan blue exclusion test.

Chromosome Analysis

About 3×10^7 cells from various S49.1A clones were incubated in growth medium in the presence of 1 μg/ml colcemid (Ciba) for 2-4 hours, centrifuged (800 x g, 5 min.) and the pellet resuspended in 5 ml of 1.12% Na citrate (37° for 30 min.), recentrifuged and the pellet fixed in 2 ml of a freshly prepared methanol: glacial acetic acid (3:1) solution (22° for 20 min.). Each sample was then centrifuged and resuspended in 0.5 ml of the fixative, dropped on an acid-cleaned,

wet slide and ignited to fix it to the slide. Preparations were stained for 30 minutes in standard Giemsa stain (Matheson, Coleman and Bell) and the number of chromosomes counted in at least five separate metaphase cells. S49.1A cells and all sub-clones examined, both steroid-sensitive and steroid-resistant, had 39-42 acrocentric chromosomes with a mode of 40, with a single exception, S49.1A.61. This steroid-sensitive sub-clone and all of its steroid-resistant progeny had 78-83 acrocentric chromosomes with a mode of 80.

Cell Growth

Cells were grown in suspension in 75 cm^2 plastic T flasks (Falcon Plastics) in Dulbecco's Modified Eagle's Medium (MEM, Grand Island Biological) supplemented with 10% heat-inactivated (56°, 1 hr) fetal calf serum (HI-FCS, Grand Island Biological) in a humidified atmosphere of 10% CO_2: 90% air. They were maintained at densities between 1×10^5 and 2×10^6 by feeding at 3-4 day intervals. Under these conditions, the population doubling time was approximately 18-20 hours.

Cloning

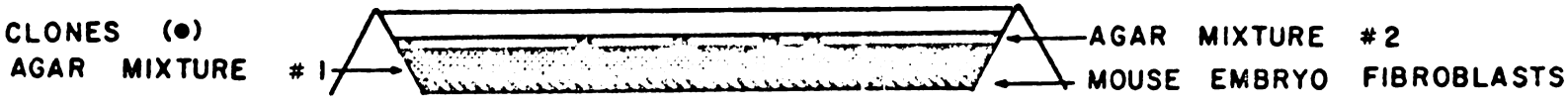
Cells were cloned in soft agar using modifications of the feeder layer methods of Pluznik and Sachs (1965) and Coffino et al., (1972). Their method has four distinct steps:

1. preparation and seeding of primary or secondary mouse embryo fibroblasts as feeder layers;

2. preparation and pouring of agar layer # 1 (Figure 3);

Figure 3.

Diagram showing the relationship of the various components of the cloning technique.


CLONES (●)
AGAR MIXTURE #1
AGAR MIXTURE #2
MOUSE EMBRYO FIBROBLASTS

3. dilution of lymphoma cells to be plated;

4. preparation and pouring of agar layer #2 containing cells to be cloned.

Our procedure differs in two respects. We seeded the fibroblasts without irradiation at very low density (5×10^4 - 1×10^5/plate) and allowed them to grow to confluence in the course of the 10 day incubation. The composition of the two agar layers differs slightly from theirs: 25 ml of agar layer #1 (enough for five plates) contains 10 ml of double strength MEM, 3 ml distilled water, 7 ml 1.8% agar (Special Noble Agar, Difco), 1 ml HI-FCS, final agar concentration 0.51%; 10.2 ml of agar layer #2 (enough for 5 plates) contains 3.6 ml double strength MEM, 1.8 ml distilled water, 1.8 ml HI-FCS, 1.8 ml of 1.8% agar, 1.2 ml of growth medium containing lymphoma cells, final agar concentration 0.31%. We plate 1.7 ml of agar mixture #2 per plate, to make the final number of lymphoma cells one-fifth the last dilution.

Isolation of Clones

After 10-14 days colonies were isolated with a sterile pasteur pipette and vigorously resuspended in 0.2-0.5 ml of a 1:1 mixture of fresh and conditioned medium in a 25 cm^2 T-flask pre-equilibrated with 10% CO_2 - 90% air. Medium was conditioned by contact with growing cells for 2-3 days, freed of cells by centrifugation (2000 x g, 5 min.) and passed through a 0.45 micron Millipore filter. The conditioned medium was mixed with an equal volume of fresh growth medium and made up to 20% serum with HI-FCS. The cultures were maintained

in a humidified CO_2 incubator, and subsequently fed with fresh growth medium. Only spherical colonies well separated from others were chosen to minimize the possibility of isolating a colony which had arisen from more than one cell.

Selection of Steroid-Resistant Variants

Selection of steroid-resistant variants required only slight changes in the standard cloning method. Cells were plated in agar mixtures 1 and 2 (Fig. 3) which contained the indicated concentration of dexamethasone (dex, Sigma), a synthetic, poorly metabolized glucocorticoid (Baxter and Tomkins, '70). In dose-response experiments the dex concentrations ranged from 5×10^{-9} to 1×10^{-7} M. In all selections to determine numbers of resistant cells in a predominantly steroid-sensitive population, the agar mixtures contained 5×10^{-7} M dex. Growth and isolation procedures were identical for cells plated in the presence and absence of dex. In all experiments cells from the same population were plated simultaneously with and without dex so that the efficiency of plating (number of colonies counted/number of cells plated) could be assessed under non-selective conditions.

Mutagenesis

Cells were treated with three mutagens, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, Aldrich Chemical) 9-aminoacridine (9-NH_2Ac, Sigma) and γ-radiation.

Cells to be irradiated were dispensed into 25 cm^2 T-flasks in 2.5 ml growth medium at a density of 5 x 10^5 to 2 x 10^6 cells per ml and irradiated with Cs^{137} at a dose rate of 275 rad equivalents per minute for 0.2 to 0.9 minute.

The methods for treatment by the two chemical mutagens were identical. The mutagens were dissolved immediately before use in dimethyl sulfoxide (Matheson, Coleman and Bell) to make a 0.1 M solution and serially diluted in growth medium until 2.5 ml contained twice the desired final mutagen concentration (range 2 x 10^{-6} to 4 x 10^{-5} M). An equal volume of cells in growth medium (2.5 x 10^5 to 2 x 10^6 cells/ml) was added and the mixture incubated in the CO_2 incubator for 2 hours. The cells were then centrifuged (800 x g for 5 minutes) and resuspended in warm growth medium at 5 x 10^5 for 2 x 10^6 cells/ml. The populations of mutagenized cells were then divided into four aliquots. The first was cloned immediately in the absence of dex (to determine toxicity of the mutagen) and in the presence of 5 x 10^{-7} M dex (to determine the effect of the mutagen on the number of resistant cells); the third and fourth were grown for 3-4 days and then cloned in the absence and presence of 5 x 10^{-7} M dex.

Steroid Binding by Whole Cells

The assay of whole cells for specific steroid binding was adapted from earlier methods (Baxter and Tomkins, 1970; Baxter et al., 1971); it is outlined in Fig. 3. Two 5-ml samples of each clone to

be tested (containing 1.25 x 10^6 to 10 x 10^6 cells) were centrifuged (800 x g, 2 min.) and the resulting pellets were resuspended in 0.5 ml of warm growth medium containing 3.5 x 10^{-9} M (^{3}H) dexamethasone (dex) (35 Ci/mmole, New England Nuclear) in the absence and presence of 5 x 10^{-5} M unlabelled competing dex. After incubation at 37° for 40 min. in a humidified atmosphere of 10% CO_2 - 90% air, the cells were centrifuged (800 x g, 2 min.,), washed quickly (within 4 minutes) with 5 ml of phosphate buffered saline (PBS: .025 M potassium phosphate, 0.1 M NaCl, pH 7.6) at 25°, recentrifuged (800 x g, 2 min.) and resuspended in 0.5 ml of ice cold PBS. A 100 µl aliquot was reserved for counting to determine specific retention of (^{3}H) dex by whole cells. The remaining 400 µl suspension was centrifuged (800 x g, 2 min.), resuspended in 0.5 ml ice cold hypotonic buffer (Medium #1, 20 mM Tricine, [N-tris-(hydroxymethyl)methylglycine, Calbiochem], 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 8.0 at 0°), lysed by freezing in liquid nitrogen and thawing at room temperature and centrifuged (1000 x g, 5 min.) to separate the lysate into a crude nuclear pellet and crude cytosol supernatant. The radioactivity in 100 µl of the latter was determined to assess specific localization of (^{3}H) dex in the crude cytosol fraction. The nuclear pellet was resuspended in 5 ml of ice cold medium #1, recentrifuged and the pellet dissolved directly in scintillation fluid and counted to determine the localization of the (^{3}H) dex in the crude nuclear pellet. Samples were counted in scintillation fluid [toluene (Mallinkrodt): Triton (Rohman-Haas): water (2840:1000:160) containing 4 gm/l Omnifluor (New England Nuclear)] in a Beck-

man LS-233 with an efficiency of 38%. The amount of specific binding was taken to be the difference between the amount of (^{3}H) dex retained in cells, or fractions comparing a sample incubated with only labeled hormone with a parallel sample containing both labeled and non-radioactive steroid (for discussion see Rousseau et al., 1972). The results were normalized to 5 x 10^6 cells to permit direct comparison of different clones.

Steroid Binding by Cell Extracts

The assessment of specific steroid binding to particle-free cytosol fractions from steroid sensitive and resistant clones was carried out as described in Rousseau et al.,(1972) except that the amount of (^{3}H) dex specifically bound to macromolecules in each sample was assayed in duplicate 75 µl samples using the DEAE cellulose filter method of Santi et al.,(1973). Filters were air-dried and counted as described above. The protein content of each cytosol sample was assayed according to Lowry et al., (1951) using bovine serum albumin as a standard.

CHAPTER THREE

THE GENETICS OF STEROID RESISTANCE

Effect of Dexamethasone on Cloning Efficiency

The S49 cells routinely cloned in soft agar with an efficiency of plating between 0.6 and 1.0. The cloning efficiency dropped dramatically when the cells were cloned in increasing concentrations of dex (Fig. 4), illustrating the lethal effect of the steroid. Figure 4 shows that at concentrations of dex greater than 5×10^{-8} M, virtually all the S49.1A cells were killed. However, if sufficiently large numbers of cells were plated in dex-containing medium a few clones grew, suggesting the presence of steroid-resistant cells in the population. In the S49.1A population (which had been cloned about three years before the present study) about 1% of the cells plated formed colonies at high steroid concentrations. Those few which appeared in steroid-containing agar were not different from those on steroid-free control plates in size, general morphology, or growth rate.

Stability of Steroid-Resistance

To determine whether the ability to grow in high steroid concentrations is a stable characteristic, colonies which appeared at 1×10^{-7} dex were isolated, grown to high cell density in steroid-free growth medium and replated in the absence and presence of 5×10^{-7} M dex. In Table 1 the results of ten such experiments, performed at different times during a two year period, are collected. The efficiency of plating of these cells was the same in the presence and absence of the

Figure 4.

Cloning of S49.1A in agar containing dex. Steroid-sensitive cells (S49.1A) were cloned as described in the Methods in the absence and in the presence of the indicated concentrations of dex. After 10 days, the number of colonies per plate was counted and is here plotted as relative efficiency of plating (efficiency of plating in dex/efficiency of plating in absence of dex). Each point is the mean of 5 plates.

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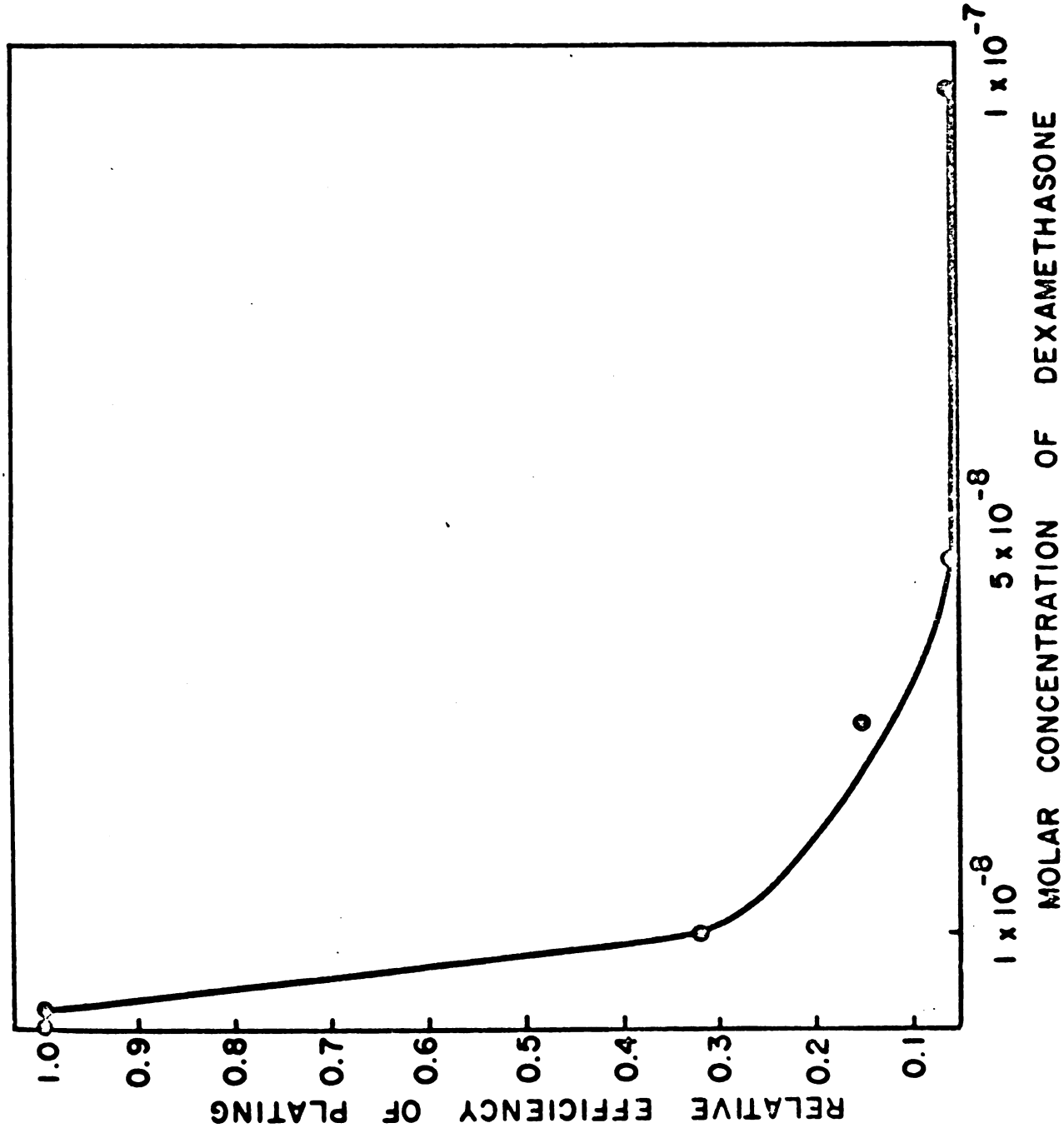


TABLE 1

Clone	Efficiency of Plating	
	O dex	5×10^{-7} M dex
S49.1A.8R	0.63	0.63
S49.1A.34R	0.28	0.32
S49.1A.35R	0.41	0.38
S49.1A.36R	0.57	0.61
S49.1A.37R	0.95	0.89
S49.1A.39R	0.62	0.50
S49.1A.3.1R	Not tested	0.63
S49.1A.3.2R	Not tested	0.32
S49.1A.3.3R	Not tested	0.71
S49.1A.3.4R	Not tested	0.72

Efficiency of plating of steroid-resistant cells. Clones which grew in the presence of 5×10^{-7} M dex were picked, grown to high density and recloned, in soft agar in the absence and presence of 5×10^{-7} M dex. After 10 days, the number of clones per plate was counted and is here expressed as the efficiency of plating. Each determination is the mean of five plates.

steroid, even though some of the clones had been grown in the absence of dex for up to seven months. This demonstrated that the resistance to steroid-killing is a stable, heritable trait.

We found that the growth of S49 cells in agar (at efficiencies greater than a few percent) required the mouse embryo fibroblast feeder layer. Since glucocorticoids are known to inhibit the growth of certain lines of mouse fibroblasts (Hackney et al., 1970), it was possible that the steroid inhibition of lymphoma cell growth over a feeder layer was due to inhibition of fibroblast function rather than to a direct effect on the S49 cells. If this had been the case, the apparently dex-resistant colonies would actually have been composed of cells which grew independently of the dex-sensitive "feeder effect". To test this possibility we examined the action of dex on the growth of steroid-sensitive and steroid-resistant colonies in suspension culture. The majority of cells in the S49 population were killed, whereas those isolated from agar containing high steroid-concentrations grew at their normal rate. Therefore, the data in Table 1, showing that the steroid-resistant cells plated with equally high efficiency in the absence and presence of dex indicate that the fibroblast "feeder effect" was not diminished by the steroid. We concluded that the dex is directly affecting the S49 cells and designated those cells which grew in its presence, steroid-resistant.

Determination of Frequency of Steroid-Resistant Cells

Since the purpose of this study was to obtain quantitative data

about the transition from steroid-sensitivity to steroid-resistance, reconstruction experiments were performed to evaluate the influence of a large number of dead cells on the plating efficiency of relatively few live ones, the situation occurring when sensitive populations were plated in high concentrations of dex and the few resistant cells survived. The presence of large numbers of dead cells increased the cloning efficiency of the live cells by about 30% (Table 2). Since our estimate of the maximum number of resistant colonies was based only on the cloning efficiency of that population in the absence of dex without this correction, we consistently underestimated the number of resistant cells by 30%.

In these experiments the absolute numbers of cells plated varied over a large range. Since the efficiency of plating might be expected to vary with density we estimated this factor for different numbers of cells. Serial dilutions of S49.1A cells were cloned in the presence and absence of 5×10^{-7} M dex. The efficiency of plating was independent of density from 2,700 to 22,000 cells per plate in the presence of dex (Fig. 5A) and from 27 to 220 cells per plate in its absence (Fig. 5B). In subsequent experiments we used cell concentrations within these ranges, so that the number of clones recovered reflected accurately the number of cells plated.

Randomness of the Transition from Steroid-Sensitivity to Resistance

Having established that the frequency of steroid-resistant cells could be estimated in a single selective step we next investigated the

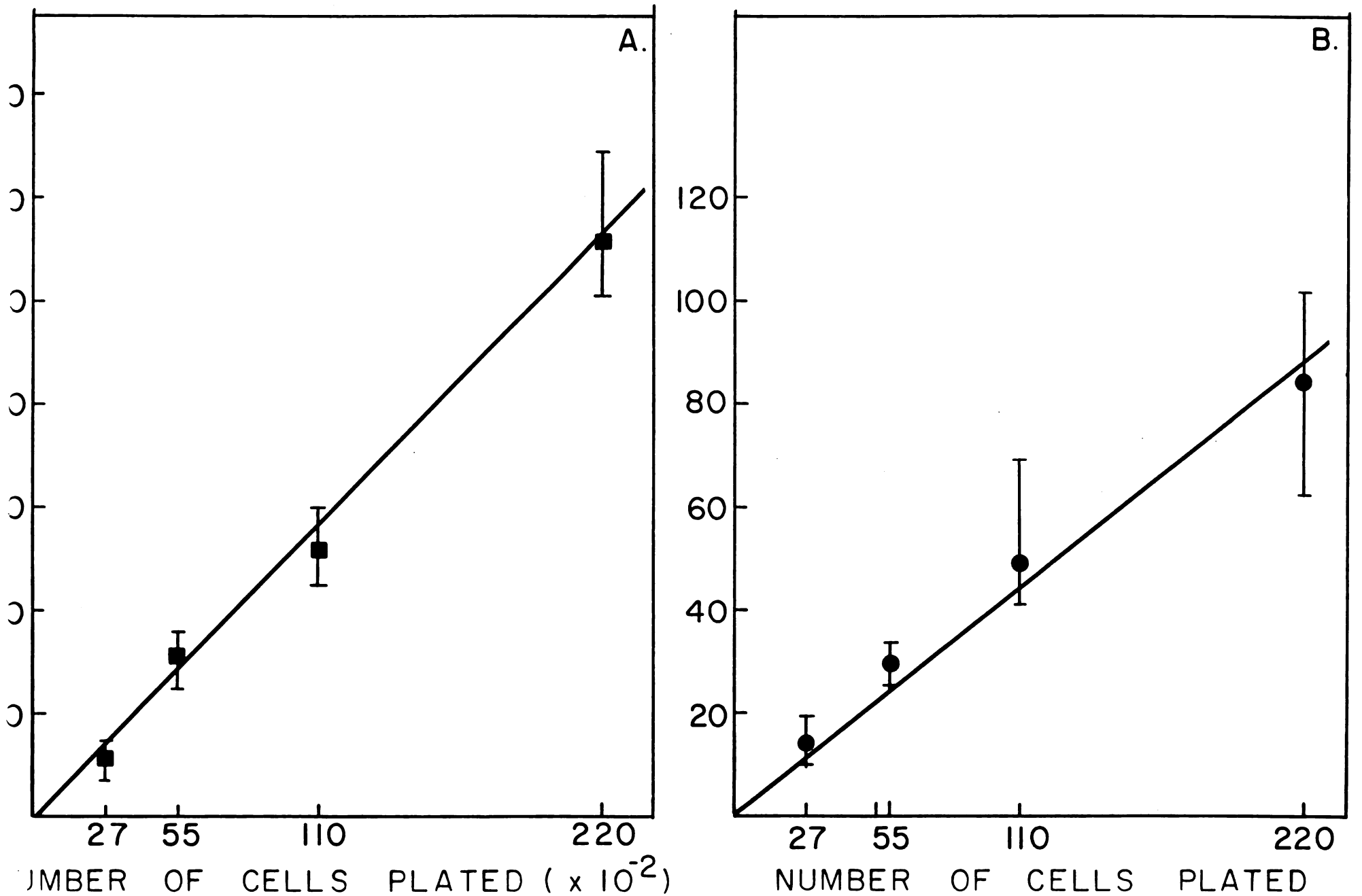
TABLE 2

Number of live cells plated	Number of dead cells plated	Plating efficiency
50	10^6	0.88
50	10^5	0.90
50	10^4	0.91
50	10^3	0.89
50	0	0.66
0	10^6	0.0

Effect of dead cells on efficiency of plating. S49.1A cell suspensions (1×10^6 cells/ml) were heated to 60° C for 5 minutes to kill all cells. The indicated number of dead cells was added to 0 or 50 live cells and the mixture cloned as described in Methods. The efficiency of plating is the mean of 5 plates.

Figure 5, A & B.

Efficiency of plating of S49.1A at various densities. Serial two-fold dilutions of steroid-sensitive cells (S49.1A) were cloned in the presence (A) and absence (B) of 5×10^{-7} M dex. After 10 days the number of colonies on each plate was counted; each point indicates the mean and range of 5 plates.


A.
27
55
110
220
JMBER OF CELLS PLATED (x 10-2)
B.
120
100
80
60
40
20
27
55
110
220
NUMBER OF CELLS PLATED

origin of the change from steroid-sensitivity to steroid-resistance. Since the transition is stably inherited, it seemed that it might result from a mutation, or from some heritable phenotypic change, perhaps related to differentiation. Since immature steroid-sensitive mouse thymocytes become steroid-resistant during their differentiation into mature immunocompetent T cells (Claman, 1972), the latter possibility seemed particularly plausible. If a phenotypic shift were involved, the steroid itself might induce it in a fraction of the population at a constant rate, analogous to the small fraction of a Pneumococcus population that is "competent" for transformation at any given time (Ravin, 1961). Alternatively, the change from steroid-sensitivity to resistance could be a random event independent of the presence of steroid, if it were a mutation, or a non-inducible phenotypic shift. A Luria-Delbrück fluctuation test (Luria and Delbrück, 1943) was performed to determine whether the transition to steroid-resistance is random. The results are tabulated in Tables 3 and 4.

One important assumption inherent in the fluctuation test is that the proportion of resistant cells in an S49 clone is so small that repeated determinations of their number are distributed according to a Poisson distribution. If this were true, the mean and variance of the distribution would be equal. Table 3 shows the results of an experiment testing this assumption. A χ^2 analysis of these data (using the variance ratio test (Simpson et al., 1960)) indicated ($p = 0.52$) that the mean and variance of the number of resistant cells from a single clone were equal. The frequency of occurrance of resistant

Table 3.

Repeated determination of numbers of resistant cells in a single clone. Clone S49.1A.1.38 was grown to a very large volume and plated repeatedly in groups of 5 plates in the absence and presence of 5×10^{-7} M dex. After 10 days, the clones were counted and the results are expressed directly as number of steroid-resistant cells per 3×10^5 cells plated. The efficiency of plating in the absence of dex was 0.9.

Table 3

S42.1A.2B	Number of resistant colonies per plate					Average number of resistant colonies per 3×10^5 cells
Plating 1	42	47	53	44	38	40.4
2	49	46	41	50	46	42.0
3	56	47	45	46	52	44.4
4	42	50	50	41	45	41.1
5	45	38	45	55	53	42.5
6	39	48	59	38	30	38.6
7	48	50	50	48	34	41.5
8	40	56	40	57	53	44.3
9	52	50	38	54	46	43.2
10	47	52	47	43	41	41.5
11	50	42	38	52	35	39.0
12	48	55	45	39	38	40.5
13	52	61	56	51	36	47.1
14	58	47	57	41		45.6
Mean						42.3
Variance						41.3
$S^2/\bar{x}$						.98

χ^2 = 12.65 with 13 d.f.

p = .52

Table 4.

Determination of numbers of resistant cells in separately isolated clones. A recently cloned steroid-sensitive sub-clone, S49.1A.1 was cloned in the absence of dex and 45 individual steroid-sensitive sub-clones picked and grown to a total of about 4×10^6 cells. Each of these was immediately cloned in pentuplicate in the presence and absence of 5×10^{-7} M dex. After 10 days the number of clones per plate was counted. The number of resistant clones per dex-containing plate is reported in columns 2-6. The efficiency of plating on steroid-free plates ranged from 0.4 to 1.00 with an average of 0.8; all but two clones fell in the range 0.6 to 1.00. Since the efficiency of plating and the actual number of cells plated on the dex-containing plates differed slightly from clone to clone, ($1.2\text{-}3.4 \times 10^5$ cells per plate), the reported average of the 3 to 5 plates is normalized to the number of resistant clones per 2×10^5 cells and corrected for the efficiency of plating of that clone on steroid-free plates to permit direct comparison.

Table 4

Experiment 1

Clone	Number of resistant clones per plate					Average number of resistant clones per 2×10^5 cells plated
S49.1A.1.1	3	2	0	4		2.2
S49.1A.1.2	1	0	0	1	0	0.4
S49.1A.1.3	0	1	0	0	0	0.2
S49.1A.1.4	0	6	4	0	2	2.2
S49.1A.1.5	67	67	53	65	65	48.0
S49.1A.1.6	6	13	9	7	9	10.0
S49.1A.1.7	8	1	6			4.4
S49.1A.1.8	0	1	0	0		0.6
S49.1A.1.9	1	3	5	5	1	2.6
S49.1A.1.10	1	1	0	0	0	0.8
S49.1A.1.11	5	4	2	4	3	3.6
S49.1A.1.12	0	0	0	0	0	0.0
S49.1A.1.15	5	6	5	4	6	6.0
S49.1A.1.16	0	1	4	3		3.0
S49.1A.1.17	0	0	0	0	0	0.0
S49.1A.1.18	2	5	4	4		4.8
Average ($\bar{x}$)						5.6
Variance (S^2)						136.4
$S^2/\bar{x}$						24.0

χ^2 384 with 15 d.f.

P<< .001

Experiment 2

Clones	Number of resistant clones per plate					Average number of resistant clones per 2×10^5 cells plated
S49.1A.1.19	0	0	0	0	0	0.0
S49.1A.1.20	3	2	5	5	4	3.0
S49.1A.1.21	3	3	5	5		4.8
S49.1A.1.24	7	3	3	3	2	2.8
S49.1A.1.25	14	9	9	26	32	13.4
S49.1A.1.26	~400	~400	~400	~400	~400	~360
S49.1A.1.27	9	12	7	16	6	7.4
S49.1A.1.28	97	83	132	133		100.0
S49.1A.1.29	4	1	0	2	3	1.8
S49.1A.1.34	0	3	1	3		1.6
S49.1A.1.35	3	3	3	0	2	2.6
S49.1A.1.36	0	0	0	0	0	0.0
S49.1A.1.37	3	10	7	6	12	8.0
S49.1A.1.38	45	41	52	64	57	40.8
S49.1A.1.39	5	5	6	13	9	6.0
S49.1A.1.41	6	8	10	13	6	7.8
Average ($\bar{x}$)						35.0
Variance (S^2)						8168
$S^2/\bar{x}$						226

χ^2 3616 with 15 d.f.

P<< .001

cells was distributed according to a Poisson distribution, validating the assumption.

In the fluctuation analysis itself, a series of sensitive clones was separately isolated and tested to determine the number of resistant cells in each clone. If the steroid uniformly induced the transition, the numbers of resistant cells in the various clones should also have been distributed according to a Poisson distribution. If, on the other hand, the transition were random, clones in which the change occurred early would have larger numbers of resistant cells than clones in which it occurred late. Furthermore, certain clones might contain no resistant cells. In consequence, if a random event gave rise to steroid resistance, the number of resistant colonies should differ widely from clone to clone and the variance would be much larger than that predicted in a Poisson distribution. Table 4 shows that the variance in the number of resistant cells per plate within a clone was small (columns 2 - 6 show actual clones per plate), whereas the variance from clone to clone (column 7) was extremely large. In one experiment, the range of numbers of resistant cells in the different clones was from 0 - 48, while in the second, this range was from 0 to approximately 400 resistant clones per 2 x 10^5 cells (too many to count accurately). The variance ratio test (Simpson et al., 1960) applied to both experiments showed that the probability that the steroid was uniformly "inducing" a transition to steroid-resistance in each of these clones was much less than .001. Therefore, steroid resistant cells arise as a result of a random event.

Rate of Generation of Steroid-Resistant Cells

To calculate the rate of this process, we assumed that the generation time of all cells, sensitive and resistant, is the same (Sibley, unpublished). The number of resistant cells in a particular clone is determined by the number of independent transitions from sensitivity to resistance and by the time of their occurrence; therefore, calculation of the rate of development of new resistant cells must account for both the appearance of new and replication of existing resistant cells in each generation. The maximal likelihood method of Lea and Coulson (1949) is one method of determining this rate, which accounts for both of these factors. Using the data in Table 4, the rate of development of new resistant cells was calculated to be 3.5×10^{-6}/cell/generation.

Effects of Mutagens on the Frequency of Steroid-Resistant Cells

The random occurrence of steroid resistance at this frequency is consistent with a mutational origin. To investigate this possibility further we examined the effects on the frequency of steroid resistance of three agents known to be mutagenic in other systems. 9-Aminoacridine ($1\text{-}2 \times 10^{-5}$ M) a frame-shift mutagen in bacteriophage T4 (Orgel and Brenner, 1961), raised the frequency of steroid-resistant cells up to 20-fold and MNNG (2×10^{-5} M), an alkylating agent in bacteria and mammalian cells (Lawley, 1968) raised the frequency 50-100-fold (Table 5). As expected, both mutagens were cytoxic and in both cases, significant increases in the frequency of resistant cells were observed only with doses of mutagen which killed greater

Table 5.

Effect of mutagens on numbers of resistant cells. Sensitive clones were isolated from the S49.1A population, grown to high density and treated with the indicated doses of mutagens as described in the Methods. The percent killing was determined from relative efficiency of plating of treated to untreated populations on steroid-free plates immediately following mutagenesis. The increased frequencies of steroid-resistant cells were found in the populations plated after 3-4 days growth in non-selective conditions, with the exception of the increases seen following irradiation of clone S49.1A.54 where the increases were observed in cells plated immediately in dex. The colonies observed in this case were small. All determinations are the mean of 4-5 plates.

TABLE 3

Clones	Treatment	% Killing	Frequency of resistant clones	Increase in stimulation over control
S49.1A.40	None		6×10^{-5}	0
	9-aminoacridine			
	1×10^{-6} M	4	12×10^{-5}	2
	1×10^{-5} M	57	75×10^{-5}	12
S49.1A.61	None		3×10^{-6}	0
	9-aminoacridine			
	4×10^{-6} M	20	8×10^{-6}	2.7
	2×10^{-5} M	74	70×10^{-6}	23
	MNNG			
	1×10^{-6} M	17	10×10^{-6}	3.3
	2×10^{-5} M	65	200×10^{-6}	66
S49.1A.63	None		$< 9 \times 10^{-7}$	0
	9-aminoacridine			
	1.5×10^{-5} M	60	32×10^{-7}	at least 4
	MNNG			
	2×10^{-5} M	80	360×10^{-7}	at least 40
	γ irradiation			
	250 rad equivalents	90	58×10^{-7}	at least 6.5
S49.1A.54	None		$< 1 \times 10^{-6}$	0
	γ irradiation			
	50 rad equivalents	6	31×10^{-5}	at least 30
	100 rad equivlents	38	23×10^{-5}	at least 23

than 50% of the population. Although cells were plated in the presence of dex either immediately after mutagenesis or after growth for 3-4 days in a non-selective medium, the frequency of steroid-resistant cells was increased only under the latter condition, similar to the experiments reported by Chu and Malling (1968).

Mutagenesis with γ-irradiation was also examined. In several experiments (Table 5) there appeared to be an increased frequency of steroid resistant cells although the results were not as consistent as with the chemical mutagens.

CHAPTER FOUR

THE MECHANISMS OF STEROID RESISTANCE

Binding of Dex by Whole Cells

Using the techniques described in the Methods, a series of steroid-resistant clones was isolated. They were characterized (as outlined in Chapter Three) as rare, stable variants of normal S49 cells, allowing the isolation of the progeny of single resistant cells and the description of the mechanism of their steroid-resistance. The early steps in cell-steroid interaction outlined in the Introduction imply that steroid-resistance might result from defects in hormone penetration, association with receptor, nuclear localization of the receptor-steroid complex, or in the subsequent reactions leading to cell death. If a series of resistant variants altered in each process could be isolated it would not only facilitate biochemical analysis but would strengthen the supposition that these events are truly involved in hormone action. Steroid-resistant cells with defects in the first two steps should exhibit diminished specific binding of low concentrations of dex. On the other hand, resistant cells with alterations in the last two processes should retain normal amounts of hormone. Since we wished to test a large number of resistant clones, we developed a quick, reliable method of distinguishing between cells with normal and diminished dex binding (Fig. 6, part A). A series of steroid-resistant clones isolated from S49.1A was characterized using this method; Fig. 7 is a histogram showing the results of these experiments.

Figure 6.

Short version of the method for determining specific retention of steroid in whole calls (part A) and in crude cell fractions (part B). Details are given in the Methods.

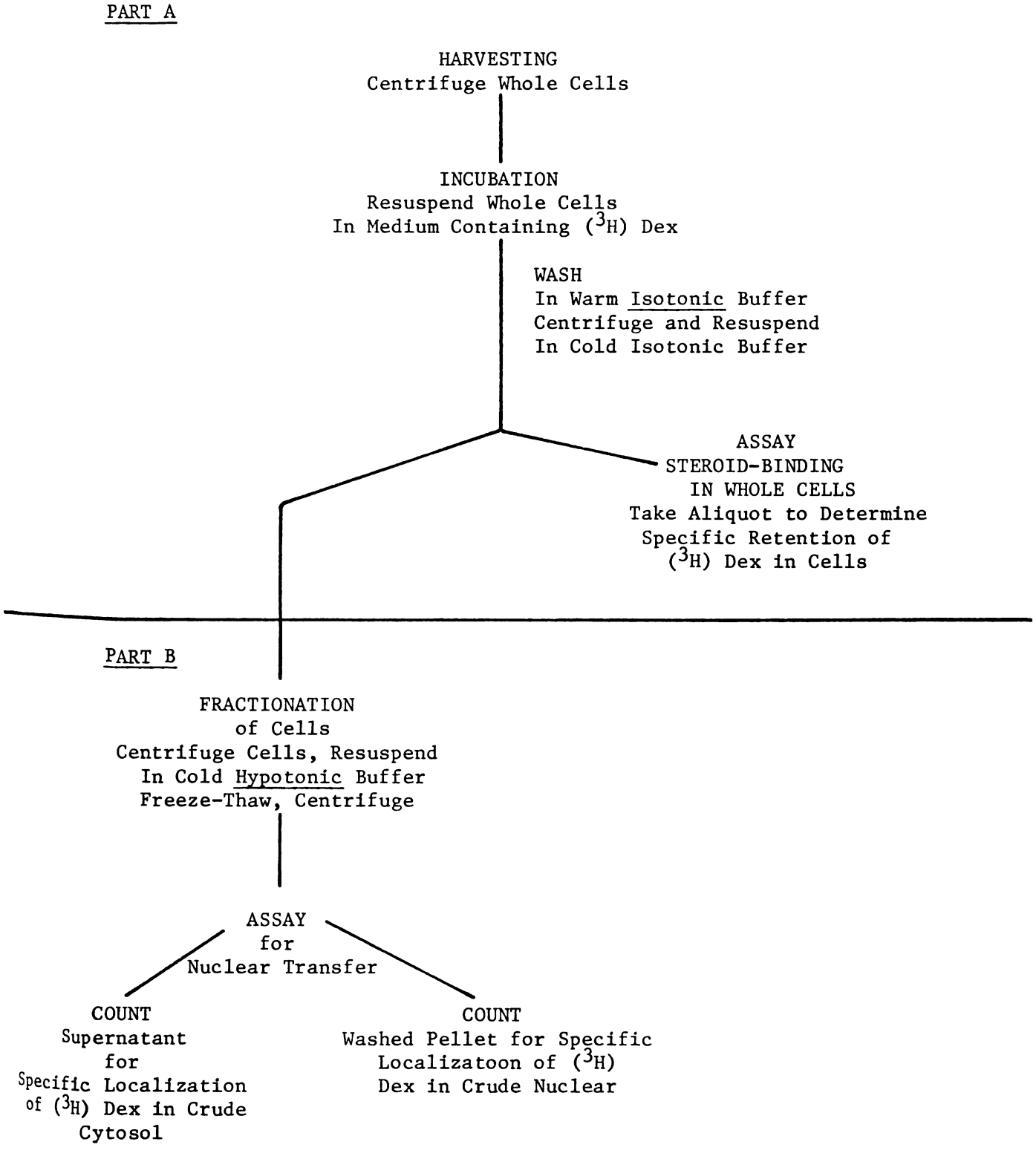
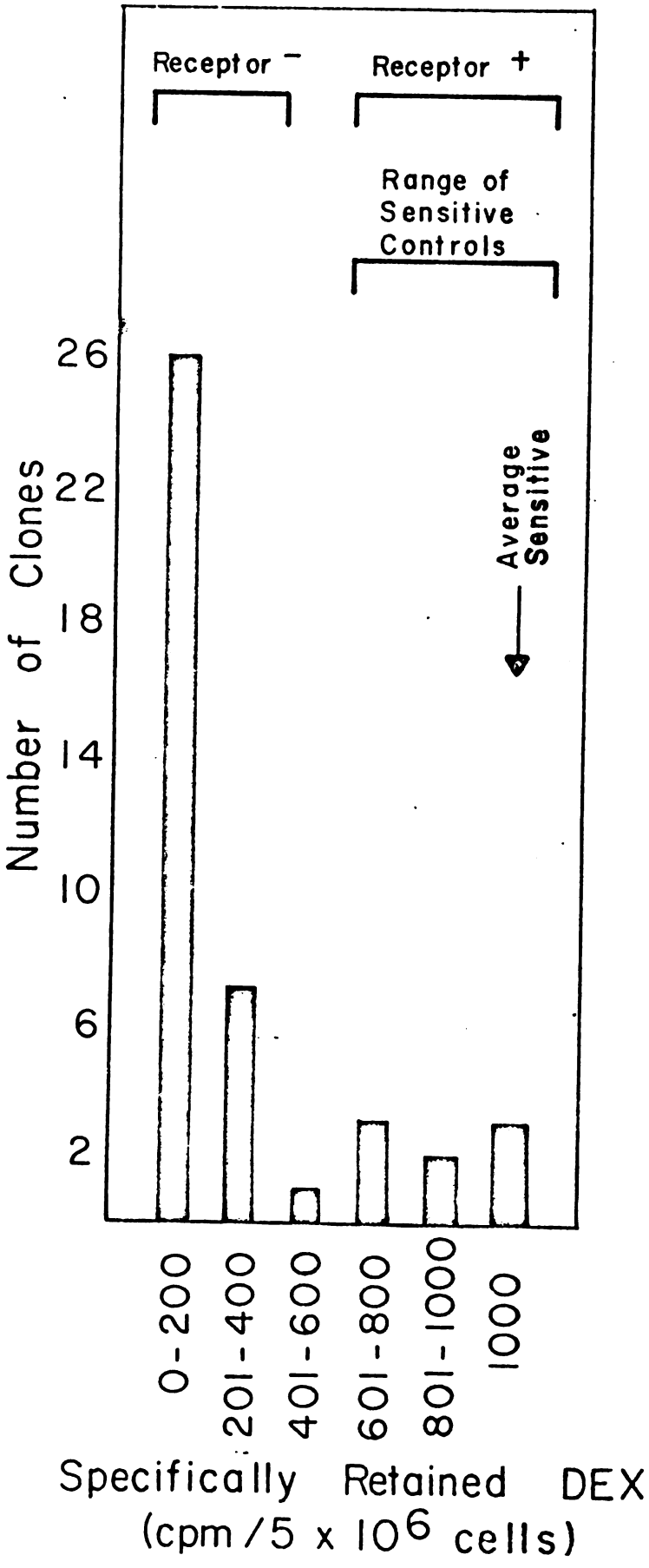

PART A
HARVESTING
Centrifuge Whole Cells
INCUBATION
Resuspend Whole Cells
In Medium Containing (^{3}H) Dex
WASH
In Warm Isotonic Buffer
Centrifuge and Resuspend
In Cold Isotonic Buffer
ASSAY
STEROID-BINDING
IN WHOLE CELLS
Take Aliquot to Determine
Specific Retention of
(^{3}H) Dex in Cells
PART B
FRACTIONATION
of Cells
Centrifuge Cells, Resuspend
In Cold Hypotonic Buffer
Freeze-Thaw, Centrifuge
ASSAY
for
Nuclear Transfer
COUNT
Supernatant
for
Specific Localization
of (^{3}H) Dex in Crude
Cytosol
COUNT
Washed Pellet for Specific
Localizatoon of (^{3}H)
Dex in Crude Nuclear

Figure 7.

Specific retention of (^{3}H) dex by steroid-resistant clones. A series of clones was selected by cloning line S49.1A in 5 x 10^{-7} M dex as described in the Methods and isolating the resulting steroid-resistant clones. These were grown to high cell density and tested for specific retention of (^{3}H) dex in whole cells as outlined in Methods and Fig. 6, part A.


Receptor −
Receptor +
Range of Sensitive Controls
Average Sensitive
Number of Clones
26
22
18
14
10
6
2
0-200
201-400
401-600
601-800
801-1000
1000
Specifically Retained DEX
(cpm/5 x 10^6 cells)

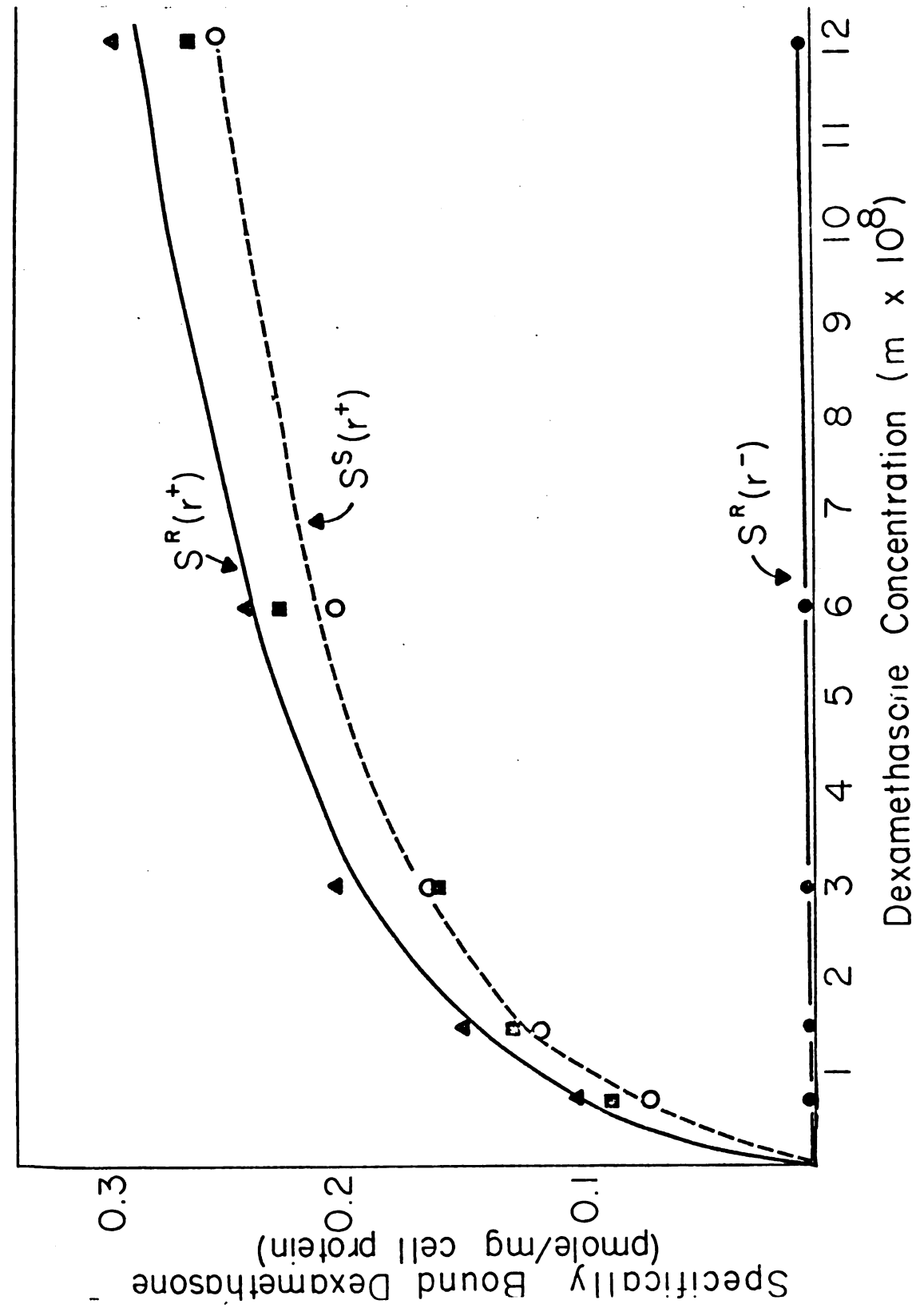
The average amount of (^{3}H) dex retained in steroid-sensitive cells under these experimental conditions was 1,709 cpm per 5 x 10^6 cells; the range was from 675 to 2,308 cpm per 5 x 10^6 cells. Thirty-three of the forty-two resistant clones tested (79%), retained little or no (^{3}H) dex (less than 400 cpm per 5 x 10^6 cells). Eight of the forty-two (19%) specifically retained (^{3}H) dex in the same range as the sensitive control (600 to > 1,000 cpm per 5 x 10^6 cells). The remaining clone which specifically retained intermediate amounts of (^{3}H) dex (400 - 600 cpm per 5 x 10^6 cells) was not further characterized.

Binding of Dex by Extracts of r+ and r- Cells

Since these steroid-resistant clones could be arbitrarily grouped into two classes, our expectation that phenotypically different types of steroid-resistant cells might arise could be tested. As indicated above, diminished steroid retention by cells could result either from impaired penetration or decreased receptor binding activity. To distinguish these possibilities quantitative cell-free steroid binding experiments were carried out with cytoplasmic extracts from two individual resistant clones in which cells retained little or no steroid (Fig. 8). As illustrated in the lowest curve, these extracts were virtually devoid of specific dex-binding activity even at the highest steroid concentration tested. These experiments show that in these clones, at least, steroid resistance resulted from a defect in the steroid-binding activity of the specific receptors. We have designated them steroid-resistant "receptor-less" S^R(r-). Furthermore, less direct evidence indicates that the remaining resistant

Figure 8.

Cell-free steroid binding activity of steroid-sensitive, receptor-containing and "receptorless" steroid-resistant clones. Particle-free supernatant fractions (cytosol) of steroid-sensitive S49.1A(0) and four steroid resistant clones, receptor-containing (S49.1A.41.16R(Δ) and S49.1A.41.22R) and receptorless (S49.1A.41.13R,0, and S49.1A.41.15R,X) were prepared as described in Methods and in Rousseau et al., (1972) and incubated for 90 min. at 0° in the indicated concentrations of (^{3}H) dex. Specifically macromolecular-bound steroid was assayed by the method of Santi et al., (1973). Each point is the mean of 3-5 experiments. These data were previously presented in (Sibley, et al., 1974).


Specifically Bound Dexamethasone
(pmole/mg cell protein)
0.3
0.2
0.1
1
2
3
4
5
6
7
8
9
10
11
12
Dexamethasone Concentration (m x 10^8)
S^R(r+)
S^S(r+)
S^R(r-)

clones with decreased dex uptake (in which cell extracts were not tested) are also r- rather than deficient in steroid penetration. This impression derives from the observation that the total cellular steroid content not bound to specific receptors (i.e., "non-specific binding" (Rousseau et al., 1972) is the same in all cells, sensitive and resistant. Clearly if steroid penetration were diminished, the non-specific association should likewise have been decreased.

In contrast, the data in Fig. 7 confirm that in certain resistant clones, the cells were able to bind normal amounts of steroid. The dex binding activity of cytoplasmic extracts from one steroid-sensitive and two resistant clones all with normal steroid retention was tested. These data are shown in the upper curves of Fig. 8. As shown, the steroid receptor activity of these steroid-resistant clones was indistinguishable from that in the normal control. These results confirmed the impression derived from whole-cell binding experiments (Fig. 7) that in these cells steroid-resistance does not result from a defect in cytoplasmic binding; a later step in hormone action must be impaired. Clones of this type have been designated steroid-resistant, receptor-containing, $S^R(r+)$.

Localization of Specifically Bound Dex in Cell Fractions

Previous experiments (Baxter et al, 1971; Roseneau et al., 1972) had suggested that nuclear localization of the receptor-steroid complex follows steroid binding in these cells just as it does in other hormone responsive systems. Therefore, we investigated whether

the complex formed when intact resistant (r+) cells were exposed to radioactive dex localized in the nucleus. Using the method outlined in Fig. 6, part B, the S^R(r+) clones were characterized on the basis of their ability to translocate steroid-receptor complex to the nucleus. These results are compiled in Table 6. In the parental, steroid-sensitive line, about half of the specifically bound (^{3}H) dex was localized in the nucleus under these conditions. Three of the eight receptor-containing (r+) resistant clones showed impaired nuclear-transfer, localizing significantly less than half of the total specifically bound steroid in the nucleus. These were designated nuclear-transfer minus, S^R(r+nt-). The remaining five r+ resistant clones transferred at least half (and in most cases significantly more) of the (^{3}H) dex to the nucleus, these were designated "deathless" S^R(r+nt+d-). Presumably, they could be clones defective in some part of the hormone response other than cytoplasmic binding and nuclear transfer.

Relative Proportions of Steroid-Resistant Phenotypes in Sensitive Clones

Since the conversion from steroid-sensitivity to resistance is a random event (see Chapter 3), the proportion of resistant phenotypes (i.e., r-, nt- and d-) in a particular clone depends on its history. For example, if the proportion of phenotypes were assayed in a population recently grown from a single sensitive cell, the preponderant resistant phenotype would be the one which had occurred earliest, since that cell had the longest time to produce progeny. On the other hand, in an "older" population the phenotypic distribution would more

Clone	localized in crude nuclear pellet: $\frac{\text{nuclear } (^3H)\text{dex}}{\text{nuclear + supernatant } (^3H)\text{dex}}$	No. of experiments	Phenotype
S49.1A (parent clone)	0.47 (0.44-0.55)	8	$S^S(r^+n^+d^+)$
S49.1A.61R	0.19 (0.18-0.21)	3	nuclear transfer minus
S49.1A.15R	0.25	1	$S^R(r^+n^-d^?)$
S49.1A.18R	0.23	1	
S49.1A.55R	0.80 (0.75-0.85)	3	"deathless"
S49.1A.57R	0.69 (0.69-0.82)	3	$S^R(r^+n^+d^-)$
S49.1A.12R	0.57	1	
S49.1A.17R	0.68	1	
S49.1A.19R	0.98	1	

Phenotypes of r^+ clones from S49.1A. A series of clones was selected by cloning the steroid-sensitive clone S49.1A in 5×10^{-7} M dex and isolating surviving steroid-resistant clones. These were grown to high density and whole cells incubated with (^3H)dex as described in the Methods and Fig. 6a and b. The partitioning of specifically bound steroid in the crude nuclear fraction was determined as the specifically bound (^3H) in nuclear pellet/total specifically bound dex in nuclear pellet + supernatant. Where more than one experiment was performed, results are shown as mean and range.

nearly represent a steady state determined by rates of both the "forward" and "reverse" transitions. These differences are shown in Table 7 where the proportions of steroid-resistant phenotypes in an "old" population, S49.1A, and several "younger" clones derived from it are given. In the steady-state S49.1A parent population, thirty-three of forty-one resistant clones were r-. In one of the derived daughter populations, S49.1A.61, the proportion of r- to r+ clones was about the same as the S49.1A (13 of 17), whereas in another daughter population, S49.1A.41, there are virtually equal numbers of r+ and r- clones.

These comparisons illustrate a number of important points. For example, none of the phenotypes detected in the steady-state population is exceedingly rare since all three were represented in the two younger clones. Furthermore, the difference in frequency of r+ resistant clones between the two daughter populations (i.e., 48% and 24% respectively) confirms the idea that the sensitive-to-resistant transition is random even in r+ cells.

A third point is that cells of one of the daughter clones, S49.1A.61 were pseudotetraploid (4s,about 80 chromosomes), whereas the parent population is pseudodiploid (2s, about 40 chromosomes). Despite this difference, the distribution of S^R phenotypes was about the same in 4s clone 61 as in the 2s parent.

The r+ resistant cells were analyzed further in terms of the distribution of intracellular steroid when whole cells were incubated

Table 7

Summary of Phenotypes of Resistant Clones

Sensitive parental	Receptor$^+$		
clone	Receptor$^-$	nuclear transfer$^-$	deathless
$S^S(r^+n^+d^+)$	$S^S(r^-n^?d^?)$	$S^S(r^+n^-d^?)$	$S^S(r^+n^+d^-)$
S49.1A	33	3	5
S49.1A.41	30	2	26 (not tested)
S49.1A.61	13	0	4

Summary of steroid-resistant phenotypes derived from various steroid-sensitive clones. S49.1A was cloned more than three years ago; the other two are recently cloned (within three months) lines, S49.1A.41 is pseudo diploid, S49.1A.61 is pseudo-tetraploid.

with dex (Table 8). Parent sensitive cells and those from both 2s and 4s resistant clones were exposed to (^{3}H) dex and fractionated as described in Fig. 6B. The results are similar to those obtained with the original parental lines (Table 6). In the present case, there were clones with decreased nuclear uptake (nt-) as well as d- clones which, as before, localized a greater proportion of the specifically bound steroid in the nuclear fraction than sensitive parent cells. We had found that treatment of S49 cells with mutagens increased the frequency of steroid resistant cells (Chapter 3). It was of interest to analyze the distribution of the resistant phenotypes after treatment of the 4s clone S49.1A.61 with nitrosoguanidine (as described in the Methods). In Fig. 9 the number of receptor-containing and -deficient cells in the control and treated populations is compared. The proportion of r- steroid-resistant cells was about the same in both populations, 13 r- of 17 in the control and 10 r- of 16 in the mutagenized cells.

Table 9 shows the subdivision of the r+ cells into nt- and d- cells in the mutagenized populations. Comparison with the unmutagenized population (Table 8) shows that mutagenesis appeared to enhance the frequency of nt- cells. There were no such cells in the untreated population, whereas 4 of 16 clones displayed the nt- phenotype after mutagenesis. Furthermore, the nt- cells isolated after nitrosoguanidine treatment appeared to transfer somewhat more dex to the nucleus than their untreated nt- counterparts. In addition, the d- cells derived from the mutagenized culture transferred a smaller

Table 8

Clone	Sensitive parental clone	Steroid sensitivity	Fraction of (^{3}H)dex localized in crude nuclear pellet: nuclear / (nuclear + supernatant) (^{3}H)dex	Number determinations	Phenotype
S49.1A.61	S49.1A	+	(0.52 (0.49-0.55)	2	$S^S(r^+n^+d^+)$
S49.1A.41.22R	S49.1A.41	-	0.19 (0.18 - 0.21)	5	nuclear transfer$^-$ $S^R(r^+n^-d^?)$
S49.1A.61.20R	S49.1A.61	-	0.90 (0.82-0.99)	2	"deathless" $S^R(r^+n^+d^-)$
S49.1A.61.22R			0.85 (0.80-0.90)	2	
S49.1A.61.27R			0.85 (0.70-0.99)	2	
S49.1A.61.38R			0.83 (0.66-0.99)	2	

Phenotypes of r^+ clones from recently isolated sensitive clones. A series of steroid-resistant clones was isolated (as described in Methods and legend to Table 6), from S49.1A.41 and S49.1A.61. Results show the mean and range of 2 to 5 experiments.

Table 9

Clone	Steroid sensitivity	Fraction of (^{3}H)dex localized in crude nuclear pellet: nuclear (^{3}H)dex / nuclear + supernatant (^{3}H)dex	Number of experiments	Phenotype
S49.1A.61.9R	-	0.20 (0.15-0.25)	3	nuclear transfer$^-$ $S^R(r^+n^-d^?)$
S49.1A.61.13R	-	0.24 (0.15-0.33)	5	
S49.1A.61.14R	-	0.25 (0.15-0.37)	5	
S49.1A.61.16R	-	0.27 (0.24-0.31)	3	
S49.1A.61.4R	-	0.51 (0.50-0.52)	2	deathless $S^R(r^+n^+d^-)$
S49.1A.61.10R	-	0.58 (0.46-0.64)	2	

Phenotypes of r^+ clones from S49.1A.61 after treatment with nitrosoguanidine. Cells of S49.1A.61 were treated with MNNG (2×10^{-5} M, 2 hours) and resistant clones isolated as described in Sibley and Tomkins (in prepration). Nuclear localization was determined as described in the Methods, Fig. 6, part B. Results are reported as the mean and range of 2 to 5 experiments.

Figure 9.

The 4s steroid-sensitive clone S49.1A.61 was cloned in the presence of 5×10^{-7} M dex, before (A) and after (B) a two hour exposure to 2×10^{-5} M MNNG. The resulting resistant clones were characterized as receptor-containing or receptor minus by the whole cell method described in the Methods and legent to Figure 6.

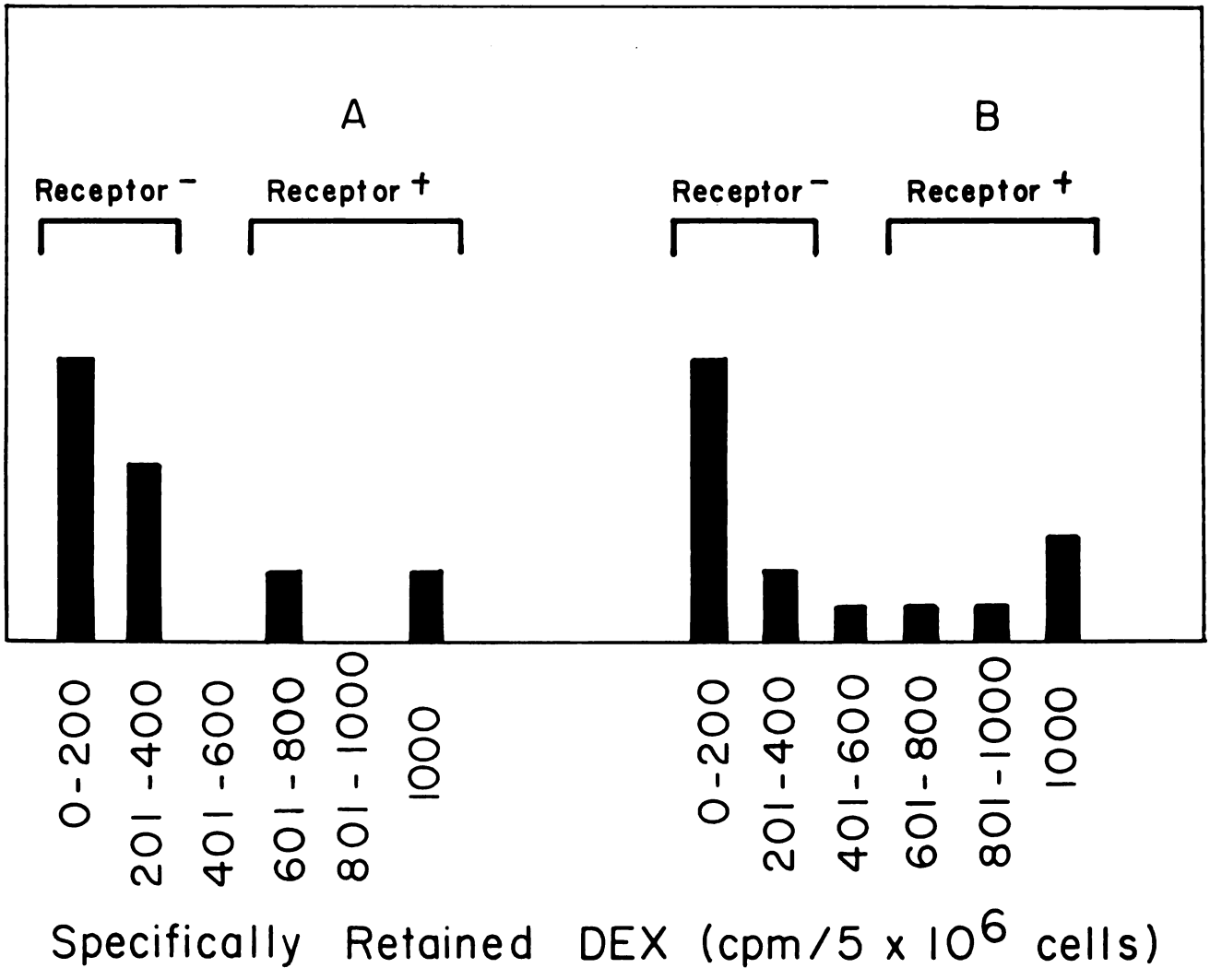
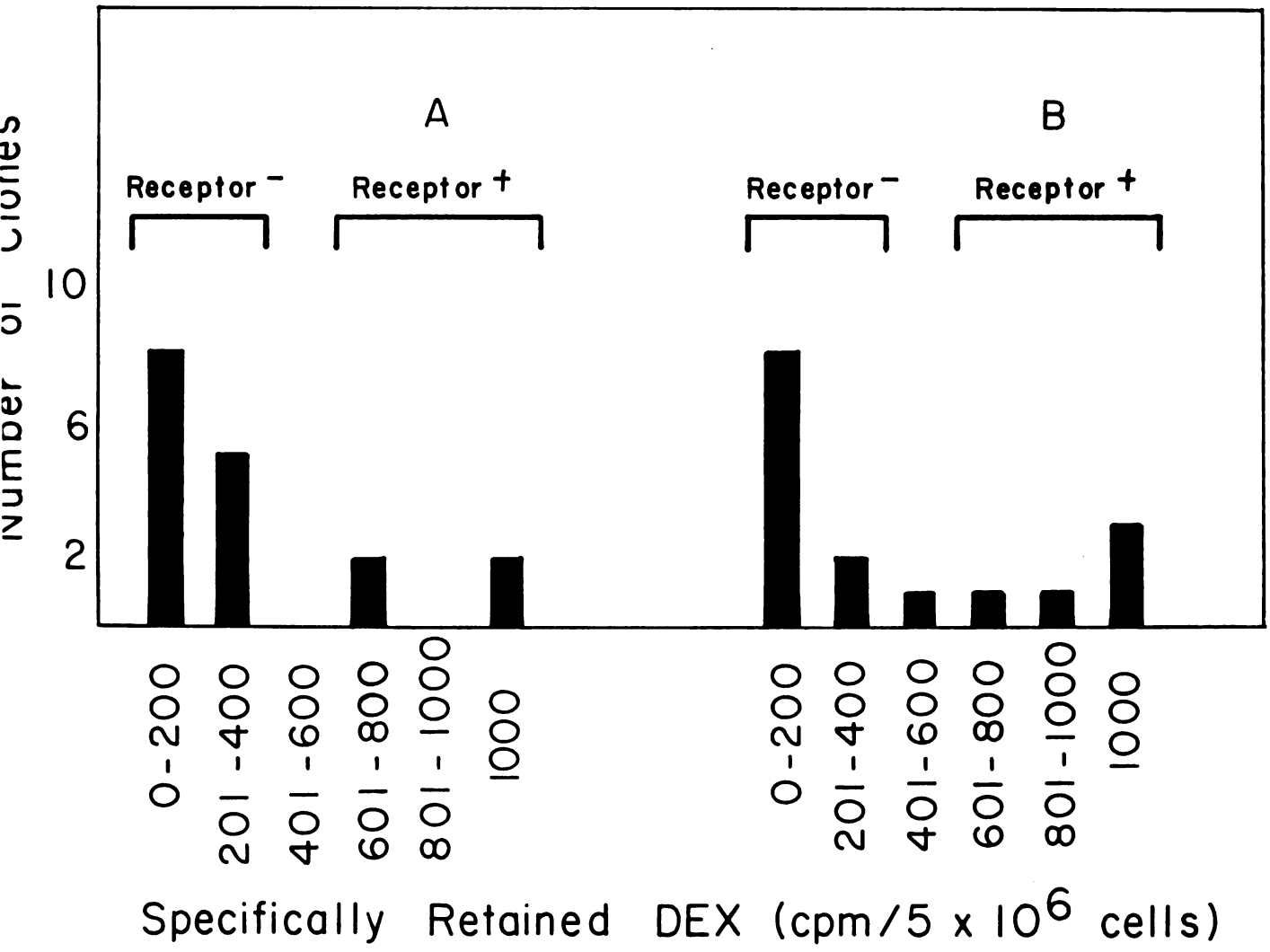

A
B
Receptor −
Receptor +
Receptor −
Receptor +
10
6
2
0-200
201-400
401-600
601-800
801-1000
1000
0-200
201-400
401-600
601-800
801-1000
1000
Specifically Retained DEX (cpm/5 x 10^6 cells)

Figure 9.

The 4s steroid-sensitive clone S49.1A.61 was cloned in the presence of 5×10^{-7} M dex, before (A) and after (B) a two hour exposure to 2×10^{-5} M MNNG. The resulting resistant clones were characterized as receptor-containing or receptor minus by the whole cell method described in the Methods and legent to Figure 6.


A
B
Receptor −
Receptor +
Receptor −
Receptor +
10
6
2
0-200
201-400
401-600
601-800
801-1000
1000
0-200
201-400
401-600
601-800
801-1000
1000
Specifically Retained DEX (cpm/5 x 10^6 cells)

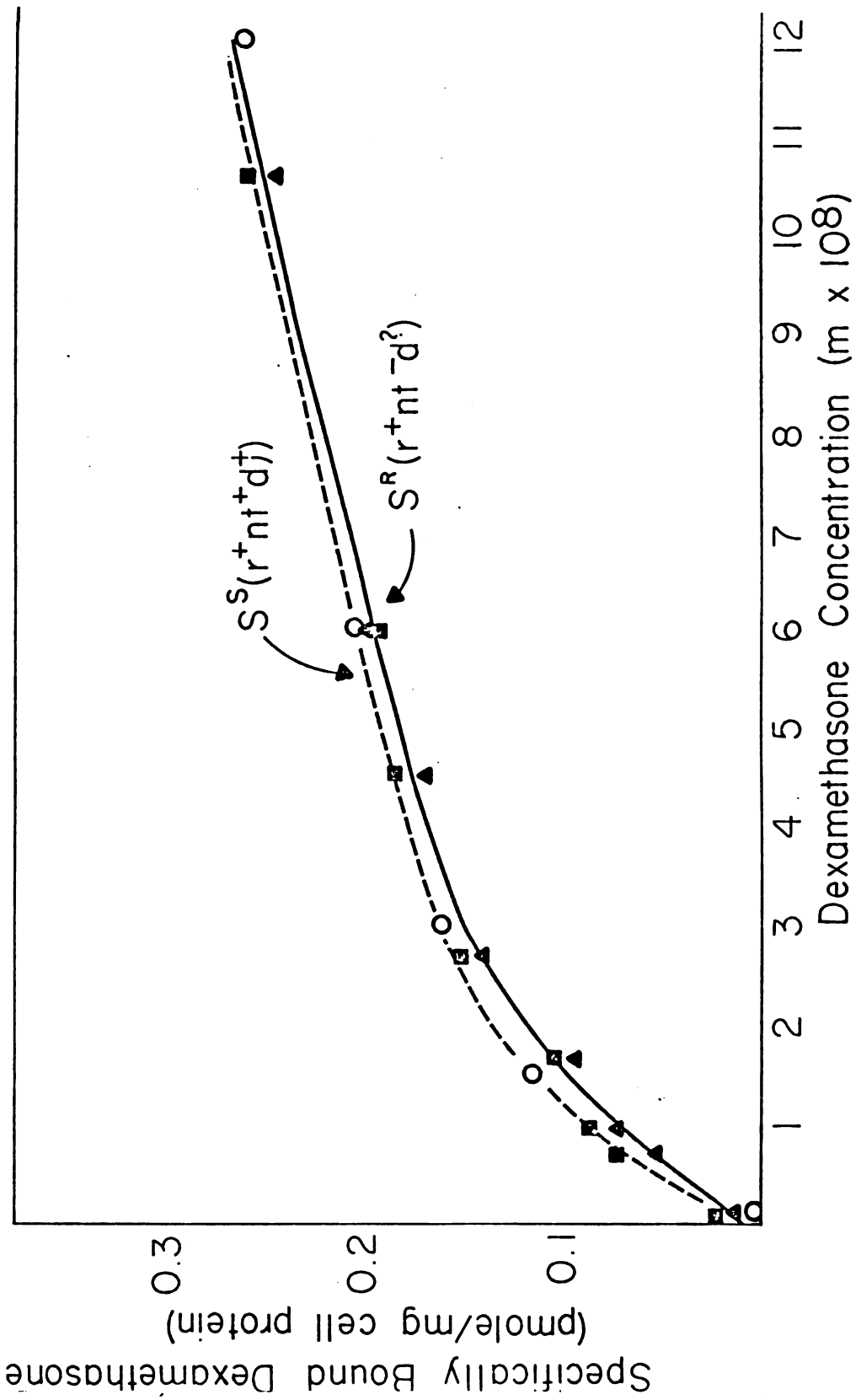
proportion of the dex to the nucleus than untreated d- cells. In fact, the extent of nuclear localization in the treated d- mutants is about the same as in the sensitive population.

Detailed Analysis of Extracts of nt- and d- Clones

One goal of this study was to isolate a set of variants in which detailed biochemical analysis would yield additional insight into the early events in steroid action. Characterization of two nuclear transfer minus and two "deathless" clones using quantitative cell-free techniques constituted a beginning of this study. As expected from the results in whole cells, extracts of these clones displayed high levels of steroid-binding activity. The binding of increasing concentrations of dex to cell extracts from sensitive controls and two nuclear-transfer minus clones is plotted in Fig. 10. The latter two populations, derived from mutagenized, pseudotetraploid (4s) cells, displayed steroid receptor activity indistinguishable from the control, exactly the result shown in Fig. 8 when r+nt- clones from pseudodiploid (2s), non-mutagenized cells were tested. Whatever the defect in the nt- cells tested thus far, it is not manifested in the cytoplasmic steroid receptor activity under these conditions. The results in Fig. 10 are expressed in terms of pmoles of bound dex per mg cell protein. Since the tetraploid cells have approximately two times the amount of protein per cell (2.29×10^{-8} gm/cell vs. 1.32×10^{-8} mg/cell), the amount of receptor per cell is also about double (0.65×10^{-8} pmole/cell vs. 0.38×10^{-8} pmole/cell). This suggests that the tetraploid cells show a dosage effect in production of receptor

Figure 10.

Cell-free steroid binding activity of steroid-resistant, nuclear transfer minus, S^R(r+nt-) and steroid-sensitive S^S(r+nt+d+) clones. Particle-free supernatant fractions were prepared from steroid-sensitive cells, S49.1A(0) and from two n- clones, S49.1A.61.13R(Δ) and S49.1A.61.14R and incubated with the indicated concentrations of (^{3}H) dex for 90 minutes at 0° as described in the Methods and in Rousseau et al., (1972). Specifically, macromolecular-bound steroid was assayed by the DEAE-cellulose filter method of Santi et al., (1973). Each point is the mean of 2 to 5 experiments.


Specifically Bound Dexamethasone
(pmole/mg cell protein)
0.3
0.2
0.1
1
2
3
4
5
6
7
8
9
10
11
12
Dexamethasone Concentration (m x 10⁸)
$S^S(r^+nt^+d^+)$
$S^R(r^+nt^-d^?)$

molecules, i.e., that a double complement of chromosomes produces twice the amount of receptor.

Extracts from the two diploid deathless clones behaved differently from the sensitive controls, both in amount of steroid binding activity and apparent affinity of their receptors for dex (Fig. 11). These parameters were more accurately estimated by replotting the binding curves using the linear method of Scatchard (1949) (Table 10). The differences between the deathless and control cells were not large. Nevertheless, it appears that the variant clones may contain receptors which, although they specifically bind dex at 0° and transfer it to the nucleus, may do so by an abnormal mechanism. Thus, the phenotype orginally designated "deathless" (where binding and transfer were normal) has not been isolated; whether the remaining d- clones display the original or this new phenotype has not yet been determined.

Figure 11.

Cell-free steroid-binding activity of steroid-resistant, "deathless" S^R(r+nt+d-) and steroid-sensitive, S^R(r+nt+d+) clones. Particle free supernatant fractions of sensitive cells, S49.1A (0) and two steroid-resistant clones classified as receptor containing, "deathless" S49.1A.55R(Δ) and S49.1A.57R were prepared and incubated with the indicated concentrations of (^{3}H) dex for 90 min at 0° as described in Methods in Rousseau et al., (1972) and the legend to Fig. 6. Specifically macromolecular bound steroid binding was assayed using the DEAE-cellulose filter method of Santi et al., (1973). Each point is the mean of 2 to 5 determinations.

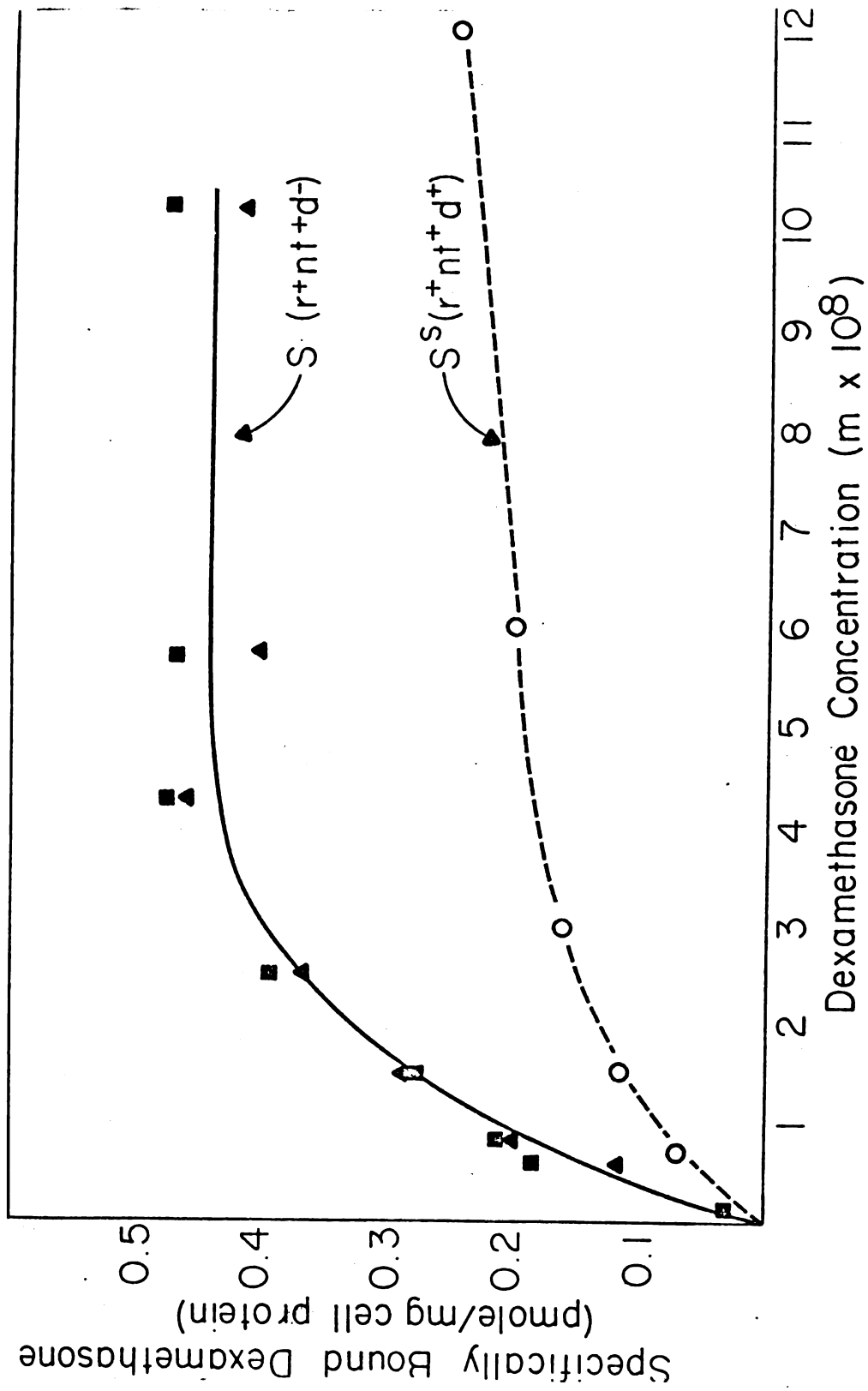

Specifically Bound Dexamethasone
(pmole/mg cell protein)
0.1
0.2
0.3
0.4
0.5
1
2
3
4
5
6
7
8
9
10
11
12
Dexamethasone Concentration (m x 10^8)
S (r+nt+d-)
S^S(r+nt+d+)

Table 10

Clone	Phenotype	Concentration of receptor-steroid complex at saturation (pmole/mg cell protein)	K_{diss} (L/mole)
S49.1A	$S^R(r^+n^+d^+)$	0.29 (0.20-0.32)	2.2×10^{-8} ($1.5 - 3.5 \times 10^{-8}$)
S49.1A.61.13R	$S^R(r^+n^-d^?)$	0.295 (0.29-0.31)	2.95×10^{-8} ($2.2-3.7 \times 10^{-8}$)
S49.1A.61.14R		0.27 (0.20-0.34)	2.2×10^{-8} ($1.2-3.1 \times 10^{-8}$)
S49.1A.41.16R		0.31 (0.26-0.33)	2.0×10^{-8} ($1.0-3.4 \times 10^{-8}$)
S49.1A.41.22R		0.33 (0.31-0.36)	1.9×10^{-8} ($1.9-2.0 \times 10^{-8}$)
S49.1A.55R	$S^R(r^+n^+d^-)$	0.44 (0.43-0.46)	7.9×10^{-9} ($7.2-9 \times 10^{-9}$)
S49.1A.57R		0.45 (0.31-0.58)	9.5×10^{-9} ($6-13 \times 10^{-9}$)

Characteristics of cell-free binding of steroid by receptors in nuclear transfer minus and deathless clones. The data in Fig. 8, 10 and 11 showing specific binding of (^{3}H)dex to receptors in particle-free cell extracts were replotted using the reciprocal method of Scatchard (1949). This allows calaculation of both the concentration of receptor-steroid complex at saturating doses of (^{3}H)dex and the affinity of these receptors for the steroid, as expressed by the K_{diss}. Values given are the mean and range of 2 - 5 experiments.

CHAPTER FIVE

DISCUSSION

In these studies we have examined the transition from steroid sensitivity to resistance in cultured lymphoma cells. On the basis of the fluctuation analysis, we have concluded that this transition is random, an impression that is strengthened by other observations. For example, the fact that 1% of the cells are resistant in a population which had been continuously grown for more than 3 years (> 1600 divisions), but in more recently cloned populations (10-30 divisions) this figure is 1000-fold lower, suggests that there is a gradual accumulation of resistant cells in sensitive populations. The difference between these two populations is slightly greater than that predicted by a random process occurring at the calculated rate (3.5×10^{-6}/cell/generation). We have no independent estimate of the reversion rate, or of any selective advantage conferred by steroid-resistance, thus this calculated rate is a composite one. Since the cells are grown in media with serum which does contain glucocorticoids (although at low, non-lethal levels, McNeill and Fleming, 1973), the resistant cells may have a selective advantage over the sensitive ones. If these substances slowed the growth of sensitive compared to resistant cells, even by a very small amount, the observed frequency of resistant cells in the "older" population could be accounted for on the basis of this selection. The fluctuation analysis clearly shows that steroid resistant cells arise randomly and presumably exist in the population prior to steroid selection. We cannot exclude the possibility that the selective agent affects the frequency

of the transition, although it seems unlikely.

The stochastic nature of the development of resistance is consistent with a mutational origin but does not establish it. This view is supported by the observations that various mutagens increase the frequency of resistant cells and that the calculated rate is similar to that of other mutation-like events in mammalian cells (Lieberman and Ove, 1959; Szybalski, 1960; Littlefield, 1964; Kao and Puck, 1967; Chu et al., 1969; Bridges and Huckle, 1970; Morrow, 1971; Fox, 1971; Albertini and De Mars, 1973). Comparing the calculated rate in our cells (3.5×10^{-6}) with haploid microorganisms (in the 10^{-7} - 10^{-8} range, Drake, 1970), it would appear that, if the change from sensitivity to resistance is indeed genetic, the mutations which lead to it are either dominant or occur in parts of the genome which are functionally haploid. We have described three phenotypes associated with steroid resistance (Sibley et al., 1974; Chapter 4). Cell hybridization experiments with this and similar cell lines (Gehring et al., 1972 and Gehring, unpublished) suggested that at least one of the three resistant phenotypes was recessive to the "wild-type", steroid-sensitive, character. These results, although fragmentary, tend to argue that steroid-resistance is recessive.

Further information about this question comes from a preliminary comparison of the frequency of the change from steroid-sensitivity to steroid-resistance in pseudo-tetraploid (4s) and in pseudodiploid (2s) populations. As noted in Table 5, dex-resistant colonies arose about

as often from the 4s (S49.1A.61) as from the 2s clones (S49.1A.40, S49.1A.63, S49.1A.54). Furthermore, steroid-resistant cells in the 2s and 4s cultures accumulated at approximately the same rate (data not shown). Finally, mutagens had effects of similar magnitude in increasing the frequency of dex resistance in clones of both ploidies. These findings argue that steroid resistance does not arise as a simple mutation in a haploid portion of the genome, since if this were the case, its frequency in 2s cells should be the square root of that in 4s cells, assuming duplication of the relevant genes. That this latter requirement is fulfilled is suggested by the fact that the 4s cells contain twice as much receptor binding activity as 2s cells (Chapter 4), demonstrating that at least these components of the hormone response have been duplicated. Therefore, while data derived from cell hybridization experiments imply that steroid resistance is recessive to sensitivity, the results of the 2s-4s comparison suggest that it is not a haploid trait. Clearly, the mechanism is not a simple one and further work like that reported by Chasin (1973) is required to resolve this paradox.

It has been suggested that certain mutation-like events in cultured somatic cells may have a physiological, rather than a genetic basis (Harris, 1971; Mezger-Freed), 1972). In this case, the characteristics of such a mechanism would be difficult to imagine. This is particularly true in view of the fact that a spectrum of different steroid-resistant phenotypes has been identified in both untreated and mutagenized 2s and 4s populations (Chapter 4). For these reasons we

favor the idea that steroid resistance arises as a result of some sort of mutational event, although of course, this conclusion can only be tentative.

A satisfactory resolution of this question is important for the treatment of steroid-sensitive malignancies. In many such cases, steroid-resistance develops during the course of drug therapy (Lippman et al., 1973). If this comes about as a result of a random physiological change in the malignant cell population, some hope might exist for reversing it. On the other hand, if the change is mutational this prospect is indeed dim. In either case, our results showing that an alkylating agent (MNNG) dramatically increased the frequency of steroid resistance, argue that the use of similar alkylating agents in combination with steroids for the treatment of such malignancies, currently a common practice, may be unwise.

Whatever the mechanisms which give rise to steroid resistant S49 populations, the transition is both stable and heritable and the system has proven useful for the analysis of cell-hormone interactions by providing variants whose response to the steroids is abberent (Sibley and Tomkins, 1974).

In the second part of this study, three types of steroid resistant variants, "receptorless", $S^R(r-)$, nuclear-transfer minus $S^R(r+nt-)$ and "deathless", $S^R(r+nt+d-)$ were identified. The relative proportions of the three types in a given population did not vary significantly as a function of how recently it had been cloned, whether it was 2s or 4s or whether it

had been mutagenized. This source of cells with abberrant responses to glucocorticoids was used to investigate the early events in steroid hormone action.

The method for determining steroid distribution in whole cells was sufficiently quick and reliable to allow the screening of large numbers of resistant clones and their assignment to three phenotypic categories. The use of small numbers of cells and low, non-saturating doses of (^{3}H) dex were important to speed and gave results which agreed within 5% for a given experiment. Therefore, the variability in specific dex binding (e.g., 675-2308 cmp/5 x 10^6 cells in S^S controls) probably reflected physiological differences in the cells from one experiment to the next, rather than methodological variation. In any case, the variability was never large enough to confuse the assignment of a particular clone to a given phenotypic category.

Our studies show that 80% of the resistant variants in a steady state population lacked specific steroid receptor activity. Therefore, it is not surprising that in other systems, steroid unresponsiveness has most often been associated with a loss of cytoplasmic steroid binding activity (Hackney et al., 1970; Kirkpatrick et al., 1971; Gehring et al., 1971; Rosenau et al., 1972), though exceptions have been noted (Gehring et al., 1972).

From a theoretical point of view, the predominance of the r- phenotype is surprising, since it is clear that binding of steroid to receptor is only one in a complicated series of reactions involved

in the hormonal induction of cell lysis (Munck et al., 1972; Makman et al., 1971). In fact the predominance of receptor variants is more striking since at least several clones characterized as r+nt-, or r+nt+d- appeared to contain receptors with altered properties. Preliminary cell-free nuclear transfer experiments (with the method of Baxter et al., 1972) using extracts from cells of the nt- clone S49.1A.41.22R have suggested that in this clone the steroid receptor has a sharply diminished capacity to associate with the nuclear acceptor sites (Gehring, pers. comm.). Furthermore, both "deathless" clones so far characterized seem to display altered receptor activity. The explanation for the disproportionately large number of receptor variants isolated is not known. It might suggest either that the genes coding for receptor activity are functionally haploid (e.g., X-linked, like the androgen receptor in mice, Gehring et al., 1971) or that mutations in other genes of the "pathway" of hormone response might be lethal.

However, as mentioned previously, simple explanations seem unable to fully account for the presently available information. The fact that the variants contain altered receptors may contribute to our knowledge of the receptor molecule itself. The r- cells were clearly deficient in dex binding activity, whereas all the extracts of nt- cells assayed thus far had normal dex-binding characteristics. On the other hand at least one of these nt- clones appeared to contain receptors which associated poorly with nuclear sites. These results are consistent with the idea that receptor molecules possess two distinct activities -- one related to steroid binding and the other to

association with the nucleus. Furthermore, extracts from d- cells bound dex more tightly than control cytosol, and some intact d- cells concentrated dex more effectively in the nucleus than normal cells. These findings suggest that (if the conversion from S^Sd+ to S^Rd- results from a single event) the steroid- and nuclear binding activities have been simultaneously altered and that both sites are part of the same molecule.

Our results are clearly consistent with the idea that the steroid-receptor complex formation is required for glucocorticoid action since receptorless cells are steroid insensitive. Furthermore, the existence of r+nt- cells indicates that nuclear localization of the complex is necessary. However, the occurrence of d- cells, in which nuclear steroid binding occurs without causing cell lysis, suggests that the presence of receptor steroid complexes in the nucleus, while necessary, is not sufficient for hormone action and that localization on specific sites in the nucleus is required.

We expect that continuation of these experiments will contribute significantly to an understanding of biological regulation by the steroids. Particularly, it seems likely that detailed biochemical studies of resistant cells will lead to a further subdivision of our initial classification of r-, nt- and d-. This should provide us with a series of variant clones in which many different steps in hormone action are altered, allowing identification of the "pathway" of response to the hormone. Since the events appear to be similar in all of the steroid-responsive tissues studied, the results would be expected to be

generally applicable to all of these systems.

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