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DRUG and HORMONE RESISTANCE in NEOPLASIA

Volume I
Basic Concepts

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FOREWORD

I am honored to be asked to write a Foreword in this text. At this point in the development of effective means for treating disseminated cancers I can think of no area that needs careful consideration and analysis more than *Drug and Hormone Resistance in Neoplasia.*

Some years ago I wrote the following:

> Cancer chemotherapy is many things. It is not just 'screening' as some seem to think, nor is it just organic chemistry, biochemistry, cell population kinetics, pharmacology, or sophisticated experimental therapeutics in model systems and in man. It is all of these things and many more, but most of all it is discovery, development, collation across disciplines, and application to man with (for good reason) a prevailing sense of urgency. We want and need and seek better guidance and are gaining it, but we cannot afford to sit and wait for the promise of tomorrow so long as stepwise progress can be made with tools at hand today.

More recently I have read that chemotherapy and adjuvant chemotherapy now cure thousands of patients bearing *certain* disseminated cancers each year. I would like to dream that improved planning with respect to selection and delivery of combinations of noncross-resistant drugs might result in significantly increased cure rates and include cancers in which only temporary responses are being achieved today. Stepwise progress is not appealing to most of us, but this is an apt description of what has happened in improving the control of about a dozen disseminated human cancers over the past two decades. If we cannot have a dramatic "breakthrough" today or tomorrow, my second choice would be continuing stepwise progress—at an accelerated pace.

New and better noncross-resistant drugs certainly are needed, but if history is repetitive new drugs also will be limited by tumor stem cells that are specifically and permanently resistant to them and often will have to be used in combination with other drugs.

The above implies my views and concerns in 1982. To me it seems clear that the inverse relationship between tumor cell burden and curability with drugs is in no small measure a reflection of the direct relationship between tumor cell burden and tumor cell heterogeneity. Large bodies of diverse experimental data support the view that the overgrowth of drug-resistant tumor stem cells during treatment is a major cause of chemotherapeutic failure. This is a stumbling block that can be attacked in a rational manner at the clinical level.

Two sentences in the Preface of this book were the first to catch my eye.

> There are few phenomena in medicine more dramatic than the production of a complete or nearly complete remission of advanced malignancy. As well, there are few things more frustrating and discouraging than to observe the relentless recurrence of a malignancy that had initially responded to treatment.

These words also describe the elation and frustration experienced over the years by some experimental oncologists I know quite well. So often we have hoped that the remissions induced in cancer-bearing animals by different treatments would carry over to humans, but that the subsequent recurrences would not. All too often both did.

What are the reasons for the recurrence of neoplasms that initially respond to cytotoxic drugs? In animal cancers they often are quite simple. If treatment is stopped after remission induction, then after recurrence a second remission often can be achieved by the same doses of the same drug or drugs. When this is possible the implication is that at cessation of treatment, and at relapse, the vast preponderance of the surviving tumor cell mix still was comprised of drug-sensitive tumor cells. On the other hand, when neoplasms regress and later recur during continuing undiminished treatment, this implies that the continuing treatment had selected to the point where, say, 50% or greater of the surviving tumor cells are resistant to the doses of the drug or

drugs being used. This has been a consistent observation repeatedly documented by harvesting the recurring tumor cells, passing them to other animals, and retesting their responsiveness to the same doses of the same drug or drugs. Neoplasms that regress and regrow during combination chemotherapy will show resistance to one or more of the drugs in the combination, but not necessarily to maximum tolerated doses of all of the drugs in the combination.

It seems to me, as to the authors of this book, that gaining both theoretical and pragmatic information regarding the phenomena responsible for the failure of anticancer drugs and hormone manipulation or hormone treatment is very important.

Finally, I am impressed with the organization of this book and the wealth of information presented in it. It will be of much value to many who are concerned with improving cancer treatment.

Howard E. Skipper, Ph.D.
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Southern Research Institute
Birmingham, Alabama

PREFACE

At the present time the most useful and effective agents for the systemic treatment of cancer are the antineoplastic drugs generally characterized as "cytotoxic" and the steroid hormones. Chemotherapeutic agents alone or in combination with hormones are capable of curing patients with a variety of disseminated malignancies. Hormones, though not curative when used by themselves, are capable of inducing significant clinical responses in those classes of tumors that are deemed hormone-dependent. Although the proportion of patients who achieve long-term remission or cure is progressively increasing, it is still apparent that the great majority of malignancies that are treated with drugs and hormones ultimately become refractory to the treatment with the resultant inevitable fatal consequences for the patient.

There are few phenomena in medicine more dramatic than the production of a complete or nearly complete remission of advanced malignancy. As well, there are few things more frustrating and discouraging than to observe the relentless recurrence of a malignancy that had initially responded to treatment. It seems self-evident that in order to improve current end results in the treatment of a great variety of neoplasms more information will be required about this process whereby tumors become resistant to the effects of systemic treatment. In these two volumes we have asked a number of investigators to review the mechanisms that are thought to underlie the development of resistance by tumors to drug and steroid hormones. From a consideration of these mechanisms we have attempted to determine to what extent current treatment protocols in a variety of malignancies are likely to be effective in overcoming resistance to treatment.

In Volume I we have reviewed the basic biological mechanisms associated with resistance in experimental systems. Similarities and differences between drug and hormone resistance are indicated. We have chosen to put some emphasis on spontaneous mutations to drug resistance as an important mechanism, as we feel that this is a process that has received insufficient attention in the past. In Volume II the clinical aspects of drug and hormone resistance are discussed. We have concentrated on malignancies of the breast, endometrium, and prostate as relevant clinical examples because these common solid tumors of adults illustrate both drug sensitivity and resistance as well as hormone dependency and hormone resistance.

In the final two chapters we have attempted to bring together a set of unifying concepts as to how resistance and treatment failure arises and from these derive inferences as to how future therapy might be more effectively directed.

The editors would like to express their thanks to the following investigators who through discussions and correspondence have provided us with many ideas and insights, and as well have permitted us to see data from studies in prepublication form: Dr. Howard E. Skipper, Southern Research Institute, Dr. Frank M. Schabel Jr., Southern Research Institute, Dr. R. W. Brockman, Southern Research Institute, Dr. J. W. Meakin, Ontario Cancer Treatment and Research Foundation, Dr. Douglass C. Tormey, Wisconsin Clinical Cancer Center.

We would as well like to thank Barbara Williams, Cynthia Wells, and Linda Wood for secretarial assistance in the preparation of these two volumes.

The editors also wish to gratefully acknowledge the support of the Cancer Control Agency of British Columbia.

N. Bruchovsky
and
J. H. Goldie

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Dr. Bruchovsky received his M.D. from the University of Toronto in 1961 and his Ph.D. in Medical Biophysics in 1966. He served as an intern at the Toronto General Hospital from 1961 to 1962 and as a resident at Parkland Memorial Hospital, Southwestern Medical School from 1966 to 1968. During this time his work with Dr. J. D. Wilson led to the proposal that dihydrotestosterone is the action form of testosterone.

Dr. Bruchovsky subsequently joined the Department of Medicine at the University of Alberta where he was promoted to the rank of Professor in 1976. In 1979 he moved to Vancouver to assume his present position with the Cancer Control Agency of British Columbia, and also as Professor of Medicine at the University of British Columbia.

Dr. Bruchovsky is a Fellow of the Royal College of Physicians and Surgeons of Canada, and a member of the Canadian Society for Clinical Investigation, the Canadian Society of Endocrinology and Metabolism, the Canadian Oncology Society, the Endocrine Society and the International Study Group for Steroid Hormones.

His present research concerns the mechanism of action of testosterone, and the control of neoplastic growth by androgens and other hormones. He has published over 50 articles on these and related topics.

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Dr. Goldie received his M.D. from the University of Toronto in 1961, and was made a Fellow of the Royal College of Physicians of Canada in 1966. His postgraduate studies in the cancer field were done at the Ontario Cancer Institute, Toronto and the Chester Beatty Institute, London, England.

Dr. Goldie has served as a member of a number of national and international committees of organizations in the cancer field. He was chairman of the investigational drug subcommittee of the National Cancer Institute of Canada and a member of the scientific grants panel of the N.C.I.C. He is also a consultant to the Division of Cancer Treatment, U.S. National Cancer Institute.

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Chapter 1

GENETIC BASIS OF DRUG RESISTANCE IN MAMMALIAN CELLS

Victor Ling

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I. INTRODUCTION

It is now recognized that drug-resistant neoplastic cells do arise during the course of the disease, and they present a major obstacle in the management of cancer. What is the origin of these cells? How rapidly do they arise? By what mechanisms do they mediate resistance? The answers to these questions will undoubtedly be required for us to be ultimately successful in chemotherapeutic treatment of cancer.

Consideration of these questions forms the theme of this chapter. The viewpoint taken is that drug-resistant neoplastic stem cells often do arise from *mutations*. Implicit in this view is that the drug-resistant phenotype is inherited and propagated; thus the well-established notion that the survival of a single drug-resistant cancer cell in vivo could prove fatal[1] is based on such a premise. This view does not exclude the importance of nongenetic events but rather it emphasizes the aspect of *hereditability* associated with genetic changes which is of overriding importance in the present context.

An attempt will be made in this chapter to present evidence that drug-resistant mammalian cells do arise from mutations and have a genetic basis. Selected examples will be taken from studies with cells in culture since they are the best characterized from this perspective. Tissue culture systems offer a number of advantages for genetic analysis which have been exploited in recent years.[2,3] For example, clonal populations can be established, rare variants can be selected, cells can be maintained under defined growth conditions, and a number of genetic manipulations can be more easily conducted. Representative mutants with different mechanisms of drug resistance will be described along with approaches for the characterization of the resistant phenotype. Three major mechanisms have been delineated and characterized: overproduction of the drug target (e.g., gene amplification), reduced drug permeability, and altered interacion of the drug with its target.

Of course, not all drug-resistant phenotypes characterized in culture systems will necessarily be represented in neoplastic cells in human; nevertheless, one can anticipate that the fundamental processes operative in generating drug-resistant mutants in culture will be universal, and that their investigation will provide tools and concepts to facilitate understanding of the in vivo situation.

II. CRITERIA ASSOCIATED WITH A GENETIC ORIGIN

Evidence in support of a genetic origin for a drug-resistant phenotype include: the phenotype is stably inherited in the absence of selection; it is spontaneously generated with a rate consistent with mutation rates in natural populations; the frequency of appearance is induced with known mutagens; an altered gene product can be demonstrated; chromosomal localization of the determinant is associated with the drug-resistance trait; and an altered gene can be demonstrated at the DNA level. Such criteria have been used in the past for differentiating between genetic changes and epigenetic modulations in bacterial systems. In mammalian cells, similar considerations applied to a number of well-characterized systems have resulted in the conclusion that most stable drug-resistant phenotypes generally do have a genetic basis.[2-6]

This conclusion is supported by data presented in Table 1. The examples presented in this table have been limited to drug-resistant mutants isolated from cultured hamster cells. The intention here is to illustrate the large repertoire of resistant phenotypes involving a wide variety of cell targets that can be obtained within a single species. Equivalent results have been obtained in other systems. This table is by no means exhaustive but some of the better characterized drug-resistant mutants are represented.

A. Frequency of Drug-Resistant Cells and Response to Mutagens

The proportion of resistant cells in a tumor or cell culture at any given moment denotes the *frequency* of the variant phenotype in question. Measurements of this sort are easily accomplished in systems where clonogenic assays have been established and a comparison of the number of surviving colonies in the presence and absence of an appropriate concentration of drug can be made. As can be seen in Table 1, in established cell cultures, typical spontaneous frequencies of mutations for a variety of stably drug-resistant phenotypes occur at 10^{-5} to 10^{-7}. These frequencies can be influenced by a number of factors such as the selective conditions used for detection (i.e., concentration of drug), age of the culture, growth conditions, and exposure to mutagens. Nevertheless, this type of data confirms that the frequency of hereditable drug-resistant phenotypes is usually low. Classical epigenetic modulations are thought generally to affect a greater proportion of a population, say 10% or more. It should be emphasized however that the frequency of a particular phenotype does not implicate any particular mechanism by which the phenotype is generated. The consideration so far simply indicates that in general, genetically based mechanisms are associated with relatively low frequencies of drug resistance in a nonselective environment.

If the frequency of a drug-resistant phenotype in a cell population is significantly increased (10 to 100-fold) after exposure to known mutagens, then this behavior is consistent with a genetic origin for the phenotype (Table 1). Nonresponse to a mutagen however is usually not definitive as not all mutagens are effective for inducing a particular phenotype. That mutagens could appreciably increase the frequency of drug-resistant mutants merits consideration from a clinical viewpoint, at least at the theoretical level, since some therapeutic agents (for example radiation and alkylating drugs) have mutagenic capability.

B. Luria-Delbrück Fluctuation Analysis of Mutation Rate

As noted above, the tacit assumption of a genetic origin for a drug-resistant phenotype is that resistant variants arise *spontaneously* at a defined *rate* in the absence of selecting agent. The Luria-Delbrück fluctuation analysis provides a means of testing the stochastic nature of generation of the phenotype.[37] This is illustrated in Figure 1 where subclonal cultures of a cell population are allowed to replicate for an appropriate defined number of generations and then each subclone is analyzed for the frequency of drug-resistant colonies. As illustrated, in situations where the resistant cells are spontaneously generated, different subclonal cultures will contain very different numbers of resistant colonies since this number will be dependent on when during the growth of the subclone to a defined size the resistant variant first appeared. Because of the spontaneous nature of generation of mutants, the fluctuation among subclonal populations will be large; thus the ratio of variance compared with the mean number of resistant colonies will be large. A rate for the generation of drug-resistant mutants can be calculated from such data.[37,38]

It is useful to keep in mind that the proportion or frequency of mutants in each subclonal population will increase as the population continues to grow until some equilibrium level is established. This is so since once a mutant is generated in a population it will also grow and divide; at the same time, new mutants are generated at a defined rate, the equilibrium level will be dependent on among other things, the mutation rate, relative growth rate of the mutants compared with the rest of the population, and reversion rate. Thus as noted above, while a frequency measurement of resistant cells in a population is not indicative of genetic origin, a rate measurement along with indications of spontaneous generation as provided by a Luria-Delbrück fluctuation test is.

Table 1
DRUG-RESISTANT HAMSTER CELL MUTANTS

Resistance to selecting drug[a]	Approximate spontaneous frequency (response to mutagens)	Luria-Delbrück analysis: mutation per cell per generation	Hybrid phenotype[b]	Altered gene product identified	DNA-Mediated transfer of resistance	Ref.
*Asparaginase	1×10^{-5} (positive)	$\sim 10^{-6}$	Dominant	Asparagine synthetase	yes	7
*β-Aspartylhydroxamate	1×10^{-7} after mutagen treatment	NR	Dominant	Asparagine synthetase	yes	8,9
*Ara A (3 classes)	4×10^{-5} (positive)	1×10^{-7}	NR	Ribonucleotide reductase (for one class)	NR	10
*Hydroxyurea (2 classes)	$\sim 10^{-5}$ (positive)	5×10^{-6}	Dominant	Ribonucleotide reductase	NR	11—13
*Ara C (2 classes)	1×10^{-6} after mutagen treatment	NR	Dominant (1 class) Recessive (1 class)	dC kinase CTP synthetase?	NR	14—16
*Melphalan	1×10^{-7} (NR)	NR	NR	Localized in the nuclear fraction	NR	17
*Methotrexate (3 classes)	3×10^{-7} (positive)	2×10^{-9} (for 1 class)	Dominant (2 classes) Recessive (1 class)	Dihydrofolate reductase drug transport	yes	18—20
*Colchicine (resistant to: adriamycin, daunomycin, vinblastine, melphalan)	5×10^{-6} (positive)	NR	Dominant	Membrane P-glycoprotein	yes	21—25
Colcemid	1×10^{-7} (positive)	NR	Dominant	Tubulin	NR	26—28
Podophyllotoxin	3×10^{-7} (positive)	4×10^{-7}	Dominant	Tubulin (probably)	NR	29
α Amanitin	$\sim 10^{-7}$ (positive)	NR	Dominant	RNA polymerase II	yes	30—32
Emetine	2×10^{-7} (positive)	5×10^{-8}	Recessive	40S Ribosomal subunit	NR	33, 34
Ouabain	1×10^{-5} (positive)	5×10^{-8}	Dominant	Membrane Na^+/K^+ ATPase	yes	3, 25, 35
Phytohemagglutinin	5×10^{-5} (positive)	1.5×10^{-6}	Recessive	Glycosyl transferase	NR	36

[a] Mutants resistant to cancer chemotherapeutic drugs are marked by an asterisk. NR = not reported.

[b] Classification of a dominant phenotype includes incomplete dominant and co-dominant phenotypes.

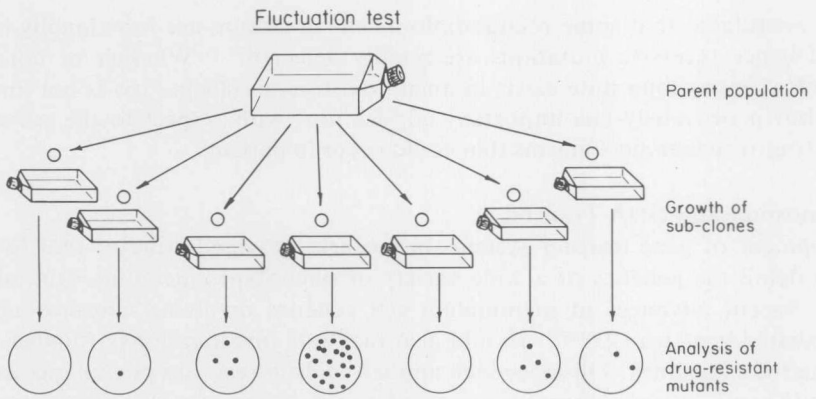

FIGURE 1. A fluctuation test to demonstrate the spontaneous generation of drug-resistant mutants.

The spontaneous mutation rate for a drug-resistant phenotype can be relatively constant for different cell lines, for example resistance to ouabain has been determined at approximately 5×10^{-8} mutations per cell per generation for mouse L-cells, hamster CHO cells, and human diploid fibroblasts[3] (see Table 1). This rate however can be significantly increased under certain circumstances such as in mutant lines where "mutator genes" have been generated,[39-41] in some virally transformed lines[42] and in certain metastatic tumor cells.[43] It is perhaps significant that the mutation rates to drug-resistance in Bloom syndrome fibroblasts are also elevated compared with fibroblasts from normal individuals.[44,45] Thus it is possible that in genetically altered neoplastic cells in vivo that the mutation rate to drug-resistance may be different from those presently observed in cultured cells.

C. Hybrid Phenotype

The technique of cell:cell hybrid formation[2,3,46] and subsequent analysis of hybrid clones offers an important genetic tool for studying the expression of a drug-resistant phenotype in a cell containing other genomes specifying different traits. A dominant or recessive nature of the resistant phenotype can be determined and this can be useful for identifying different mechanisms of resistance (c.f., Table 1, and discussion of mutants resistant to methotrexate IIIB). In the case of recessive markers, hybrids can be used to delineate complementation groups revealing different alterations giving rise to a similar phenotype. With dominant markers, hybrids can be used to study the effects of gene dosage or different genomic combinations on the expression of a resistant phenotype. Chromosome loss occurs in hybrid cells especially with crosses between cells of different species.[46,47] Such reduced hybrids have been used to map the determinants of drug-resistant phenotypes on specific chromosomes. Thus analysis of a variety of hybrid cells has provided strong evidence for a chromosomal origin and a genetic basis for many drug-resistant phenotypes.

It is pertinent to ask whether a particular drug-resistant phenotype is dominant or recessive since in principle, recessive mutations would not be expressed at frequencies readily observable in fully diploid cells where two or more mutations at the same locus would be required before the phenotype could be expressed. In this context it is significant that highly malignant tumors are often aneuploid with a tendency to maintain a hyperdiploid state.[48] However, from Table 1, it is seen that many phenotypes resistant to chemotherapeutic drugs identified in culture cells are dominant, and in theory at least, they can be expressed in polyploid cells as a result of only a single mutation. It

has been postulated that some pseudodiploid cells in culture are functionally hemizygous and hence recessive mutations are readily detected.[5,49] Whether or not such a functionally hemizygous state exists in aneuploid tumor cells in vivo is not known at present, but it obviously has important implications with respect to the variety and types of drug-resistant mechanisms that could occur in patients.

D. Chromosome and DNA Transfer

Development of gene transfer systems in bacteria provided a major tool for elucidating in detail the genetics of a wide variety of phenotypes including drug-resistant markers. Recent advances in mammalian cell genetics involving chromosome- and DNA-mediated gene transfer[50-53] should also facilitate investigations of higher organisms along the same lines. This approach applied to drug-resistant phenotypes in mammalian cells provides unequivocal evidence of a genetic basis for these phenotypes. As can be seen in Table 1, such evidence have been obtained in a number of well-characterized systems. In practice dominant resistant phenotypes are easier to work with since recipient cells expressing the appropriate marker can be selected directly. Also, it has been observed that unlinked markers in the genome are generally not cotransferred, thus coexpression of different phenotypes in a transformant is likely due to the pleiotropic nature of the mutant gene (for example, multiple resistance to different drugs expressed in colchicine-resistant mutants IIIC).

The ability to transfer a drug-resistant phenotype via DNA also provides an approach for isolating the gene(s) in question by molecular cloning.[53,54] The potential of using purified genes for investigating drug resistance in mammalian cells has not yet been fully exploited.

E. Summary

It is quite clear from the data presented in Table 1 that a very wide variety of stable drug-resistant phenotypes with properties completely consistent with a genetic origin can be isolated from mammalian cells. Although consideration of drug-resistant lines has been limited here only to well-characterized cells in culture, it would be surprising if the basic mechanisms generating such mutants were not also operative in cells in vivo. Of course the type and variety of mutants represented could be different, but the process of generating variants from mutations and enrichment of subpopulations by selection would be universal.

III. PROPERTIES OF DRUG-RESISTANT MUTANTS

A. General Considerations

In this section, properties of mutants resistant to some anticancer drugs are considered along with approaches used to delineate their mechanisms of resistance. With respect to the drug-resistant phenotype itself, three aspects deserve further discussion.

1. Stability

The mutants listed in Table 1 all display stable drug-resistant phenotypes, maintaining their resistance in the absence of selecting agents for one month or longer in culture. This aspect is consistent with the heritability of genetic mutations. Unstable resistant phenotypes are sometimes observed and investigation of the origin and mechanism of such phenotypes is more difficult. Instability could of course implicate an epigenetic mechanism; however, in our experience it is not uncommon to observe that in the absence of drug, drug-resistant mutants have appreciably slower growth rates. Thus if the "fitness" of drug-sensitive revertant cells for growth is better than